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(57) Abstract

The present invention is directed towards NH_2-terminally truncated analogs of three human chemokines: MCP-3, RANTES and MIP-1α having highly potent anti-inflammatory activity and anti-autoimmune activity. The present invention is also directed to inhibiting the biological activities of three native, mammalian chemokines: MCP-3, RANTES and MIP-1α. The present invention is further directed to treating inflammatory diseases and autoimmune disorders such as rheumatoid arthritis, for example. The present invention is also directed to pharmaceutical compositions comprising NH_2-terminally truncated chemokine analogs.

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MCP-3, RANTES AND MIP-1ALPHA RECEPTOR ANTAGONISTS

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FIELD OF THE INVENTION

This invention relates to NH_2-terminally truncated analogs of Monocyte Chemoattractant Protein-3 (MCP-3), RANTES and Macrophage Inflammatory Protein-1α (MIP-1α) and compositions containing same and methods of employing said compositions for treating inflammatory diseases as well as autoimmune disorders.

BACKGROUND OF THE INVENTION

Regulated on Activation, Normal T-Cell Expressed and Secreted (RANTES), Monocyte Chemoattractant Protein-3 (MCP-3) and Macrophage Inflammatory Protein-1α (MIP-1α) are inflammatory mediators, characterized as chemotactic cytokines or chemokines. The term "chemokine" reflects the ability of these mediators to induce directed migration of several types of leukocytes, including monocytes, lymphocytes, basophils and eosinophils into sites of inflammation.

RANTES, MCP-3 and MIP-1α are collectively known as "CC" chemokines because the first two cysteines in each molecule are adjacent. The CC

chemokines contain disulfide bridges. The CC chemokines have been implicated in a number of allergic and chronic inflammatory diseases such as arthritis and various lung diseases. In such conditions, early monocytic infiltration of monocytes, T-lymphocytes and other leukocytes may be a key event in the progression of the disease.

Multiple receptors mediate the functional activities of the CC chemokines. Five receptors have been identified and their polypeptide sequences deduced from cDNA clones. These are: chemokine receptor CCKR-1, which binds MIP-1α, RANTES and MCP-3 (Gao, et al. (1995) J. Exp. Med. 177:1421-1427); CCKR-2, which binds MCP-3 and MCP-1 (Franci, et al. (1995) J. Immunol. 154:6511-6517); CCKR-3, which binds to RANTES, MCP-3 and Eotoxin (Kitaura, et al. (1996) J. Biol. Chem. 271:7725-7730); $CCKR_4$, which binds to MCP-1, RANTES and MIP-1α (Hoogawerf, et al. (1996) Biochem. Biophy. Research Communication 218:337-343), $CCKR_5$, which binds to MIP-1α/β and RANTES (Raport, et al. (1996) J. Biol. Chem. 271:17161-17166).

The complete amino acid sequences of RANTES, MCP-3 and MIP-1α were described in Clark-Lewis, et al. (1995) J. Leukocyte Biol. 57:703-711. RANTES comprises a 68 amino acid polypeptide, MCP-3 comprises a 76 amino acid polypeptide and MIP-1α comprises a 70 amino acid polypeptide.

It has been suggested that substances that are capable of blocking the effects of RANTES, MCP-3 and/or MIP-1α would be useful to moderate or inhibit various inflammatory and allergy reactions. The

patent application of Wells, et al. filed under the Patent Cooperation Treaty and published on June 13, 1996 under WO 96/17935, describes modified RANTES polypeptides possessing antagonistic activity. The polypeptides of Wells, et al. comprise the addition of amino acids to the N-terminal end of RANTES so that the number of amino acids in the antagonists described therein have a longer chain length than the corresponding native RANTES.

Masure, et al. (1995) J. Interferon and Cytokine Res. 15:955-963, describe the expression of mutant MCP-3 in Pichia pastoris which possesses three additional NH_2 terminal amino acids. Masure, et al. characterized mutant MCP-3 as an MCP-3 receptor antagonist capable of competing with synthetic MCP-3.

Heretofore, no one knew that NH_2 terminally truncated chemokine analogs of RANTES, MCP-3 or MIP-1α possessed significant antagonistic activity with very high receptor binding affinities. However, the present inventors have not only discovered the antagonistic effects of truncated RANTES, MCP-3 and MIP-1α, but also have discovered that the analogs are useful for treating inflammatory diseases and autoimmune disorders, e.g., rheumatoid arthritis. The inventors have also discovered that the analogs competitively bind and cross-bind to several different receptors. Inflammation involves the infiltration of multiple cell types that occurs through the interaction of different chemokines with distinct functional receptors. The inventors have further discovered that blocking the infiltration of multiple

effector cells is highly effective in breaking the inflammation and autoimmune cycle.

SUMMARY OF THE INVENTION

The present invention is directed towards NH_2-terminally truncated analogs of three human chemokines: MCP-3, RANTES and MIP-1α having anti-inflammatory activity and/or anti-autoimmune activity. The present invention is also directed to inhibiting the biological activities of three native, mammalian chemokines MCP-3, RANTES and MIP-1α. The present invention is further directed to treating inflammatory diseases and autoimmune disorders such as rheumatoid arthritis and multiple sclerosis, for example. The present invention is also directed to pharmaceutical compositions comprising NH_2-terminally truncated chemokine analogs.

One aspect of the present invention is directed to an analog of mammalian MCP-3 lacking NH_2-terminal amino acids corresponding to amino acid residues 1-6, 1-7, 1-8, 1-9 or 1-10, of MCP-3, having substantial homology to the native MCP-3 molecule.

Another aspect of the present invention is directed to an analog of mammalian RANTES lacking NH_2-terminal amino acids corresponding to amino acid residues 1-5, 1-6, 1-7, 1-8, and 1-9 of RANTES, having substantial homology to the native RANTES molecule.

A further aspect of the present invention is directed to an analog of mammalian MIP-1α lacking NH_2-terminal amino acids corresponding to amino acid

residues 1-9 or 1-10, having substantial homology to the native MIP-1α molecule.

A still further aspect of the present invention is directed to a method of inhibiting the biological activity or the in vivo biological activity of native MCP-3, RANTES and MIP-1α comprising adding to the native MCP-3 RANTES and MIP-1α (if in vitro) or if in vivo administering to a host, e.g., mammal (for example, human) a therapeutically effective amount of an analog of MCP-3, RANTES or MIP-1α for a time and under conditions sufficient to inhibit the biological activity of the native molecules.

Another aspect of the present invention is directed to a method of treating inflammatory diseases in a mammal suffering from said diseases comprising administering to said mammal a therapeutically effective amount of the analog of the present invention.

A further aspect of the present invention is directed to a method of treating autoimmune disorders in a mammal comprising administering to a mammal in need thereof a therapeutically effective amount of an analog of the present invention.

Another aspect of the present invention is directed to pharmaceutical compositions comprising an antagonistically effective amount of one or more of the aforementioned analogs and a pharmaceutically acceptable carrier.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1A is a graph showing NH_2-terminal truncation of RANTES and MCP-3 resulted in a loss of chemotactic activity on THP-1 cells.

Figure 1B is a graph showing the NH_2-terminal truncated RANTES analogs (RANTES(6-68), RANTES(7-68), RANTES(8-68), RANTES(9-68), and RANTES(10-68)) had no detectable activity.

Figure 1C is a graph showing truncation of the residues 1-9 of MIP-1α (MIP-1α (10-70)) reduced activity by more than 95% of the native MIP-1α, further truncation resulted MIP-1α (11-70) which completely lost chemoattractant activity on THP-1 cells.

Figure 2A is a graph showing RANTES antagonist activity of the RANTES(9-68) analog titrated at the indicated concentrations against RANTES and MCP-3 in a chemotaxis assay using THP-1 cells.

Figure 2B is a graph showing MCP-3 antagonist activity of the MCP-3(10-76) analog titrated at the indicated concentrations against MCP-3 and RANTES in a chemotaxis assay using THP-1 cells.

Figure 2C is a graph showing the inhibition of MIP-1α induced (10 nM or 10^{-8}M) THP-1 migration by MIP-1α(10-70) and MIP-1α(11-70).

Figure 2D is a graph showing the inhibition of RANTES-induced (10 nM or 10^{-8}M) THP-1 migration by MIP-1α(10-70) and MIP-1α(11-70).

Figure 3A is a graph showing RANTES antagonist activity of RANTES(9-68) titrated at the indicated concentrations against RANTES-induced and MCP-3-induced N-acetyl-β-D-glucosaminidase release using human blood monocytes.

Figure 3B is a graph showing MCP-3 antagonist activity of MCP-3(10-76) titrated at the indicated concentrations against MCP-3-induced and RANTES-induced N-acetyl-β-D-glucosaminidase release using human blood monocytes.

Figures 3C and 3D are graphs showing MIP-1α antagonist activity of MIP-1α(10-70) and MIP-1α(11-70) titrated at the indicated concentrations against MIP-1α-induced and RANTES-induced-N-acetyl-β-D-glucosaminidase release using human blood monocytes. The concentration of MIP-1α and RANTES used was 30 nM.

Figures 4A and 4B are graphs showing competitive binding to THP-1 cells of unlabeled RANTES and RANTES(9-68) titrated at the indicated concentrations in the presence of 4 nM labeled RANTES (A), and 4 nM labeled MCP-3 (B).

Figures 4C and 4D are graphs showing competitive binding to THP-1 cells of unlabeled MCP-3 and MCP-

3(10-76) titrated at the indicated concentrations in the presence of 4 nM labeled MCP-3 (D) and 4 nM labeled RANTES (C).

Figure 4E is a graph showing competitive binding to THP-1 cells of unlabeled MCP-3 and MCP-3(8-76), MCP-3(9-76), MCP-3(10-76) and MCP-3(11-76) titrated at the indicated concentrations in the presence of 4 nM labeled MCP-3.

Figure 4F is a graph showing competitive binding to THP-1 cells of unlabeled RANTES and RANTES (6-68), RANTES (7-68), RANTES (8-68), RANTES (9-68) and RANTES (10-68) titrated at the indicated concentrations in the presence of 4 nM labeled RANTES.

Figure 5 is a graph showing competitive binding to THP-1 cells of unlabeled MIP-1α and MIP-1α(10-70) and MIP-1α(11-70) titrated at the indicated concentrations in the presence of 4 nM labeled MIP-1α.

Figure 6 is a chart summarizing the binding and antagonist activities of MIP-1α, RANTES and MCP-3 analogs.

Figure 7 is a graph showing competitive binding to THP-1 cells of unlabeled RANTES and RANTES(9-68) and RANTES(9-68, P9T) titrated at the indicated concentrations in the presence of 4 nM labeled MCP-1.

Figure 8 is a graph which shows that human RANTES(9-68) was able to bind to the receptor on mouse peritoneal macrophages. Native human RANTES did not significantly bind to mouse peritoneal macrophages.

Figures 9A and 9B are graphs showing that daily i.p. injections of RANTES (9-68) inhibited the swelling and the incidence of arthritis in treated mice.

Figure 10 is a chart showing untreated control animals and animals administered a peptide control developed rheumatoid arthritis, substantial infiltration of mononuclear cells into the synovial tissue, extensive hyperplasia of the synovial lining, pannus formation and bone and cartilage damage. In contrast, animals treated with RANTES(9-68) exhibited no invasive pannus, no bone or cartilage damage and insignificant inflammatory infiltration.

Figure 11 is a graph which shows that human MCP-3 (9-76) was able to bind to the receptor on mouse monocytes. Native human MCP-3 did not significantly bind to mouse monocytic cells.

Figure 12 is a graph showing that daily i.p. injections of MCP-3 (9-76) inhibited the swelling and the incidence of arthritis in treated mice.

DETAILED DESCRIPTION OF THE INVENTION

One aspect of the present invention is directed to NH_2-terminally truncated analogs of three human chemokines, MCP-3, RANTES and MIP-1α, useful for treating inflammatory conditions and autoimmune disorders. Inflammatory conditions contemplated by the present invention include both acute and chronic inflammatory diseases. Examples include, but are not limited to, arthritis, asthma, colitis, psoriasis, atheromas and the like. Examples of autoimmune conditions include rheumatoid arthritis and multiple sclerosis and the like. As defined by the present invention an NH_2-terminally truncated chemokine analog acts as an antagonist to a corresponding native chemokine. The antagonistic activity of the chemokine analogs of the present invention includes inhibition of biological activity induced by corresponding native chemokines.

In the application, the products of the present invention are referred to by various terms, including "analogs of the present invention", "NH_2-terminally truncated analogs", "polypeptides of the present invention", "antagonists of the corresponding chemokines", etc. are used interchangeably and denote equivalent expression.

As defined by the present invention biological activity refers to the biological activity of the native chemokines, as measured by standard assays, including but not limited to receptor binding, chemotaxis, calcium mobilization and exocytosis

characterized by N-Acetyl-β-D-glucosaminidase release and elastase release.

Preferred MCP-3 analogs of the present invention include: MCP-3 (7-76), (8-76), (9-76), (10-76) and (11-76). Preferred RANTES analogs of the present invention include: RANTES (6-68), (7-68), (8-68), (9-68) and (10-68). Preferred MIP-1α analogs of the present invention include: MIP-1α (9-70), (10-70) and (11-70).

As defined by the present invention any reference by number to an amino acid in an analog, e.g., MCP-3 analog will be a reference to the corresponding residue number from the amino acid sequence of the native molecule shown in Table 1. For example, where the first 7, 8, 9 or 10 amino acids of MCP-3 are truncated (as is the case for the MCP-3 analogs shown in Table 1) the analogs will be referred to MCP-3 (8-76), MCP-3 (9-76), MCP-3(10-76) and MCP-3(11-76), respectively.

The analogs of the present invention comprise an amino acid sequence that is identical to the corresponding region of the native molecule, or a polypeptide fragment having a region that is substantially homologous to all or part of a region of the corresponding native molecule while still maintaining the antagonist activity to the corresponding native molecule. The analogs of the present invention maintain the ability to competitively bind at the receptor site of the corresponding chemokine. Analogs which are "substantially homologous" to all or part of a region

of the native molecule are characterized as having from 1-10 amino acid deletions, additions or substitutions that do not result in an analog losing its ability to compete with a native chemokine for binding to a chemokine receptor while still retaining the antagonist activity. It is preferred that the MCP-3 analogs of the present invention are at least about 80% homologous to native MCP-3 and can have as much as 100% homology to native MCP-3, inclusive. More preferably, MCP-3 analogs of the present invention are at least about 85% homologous to native MCP-3. Still more preferably, MCP-3 analogs of the present invention are at least about 90% homologous to native MCP-3. Most preferred MCP-3 analogs of the present invention are at least about 95% homologous to native MCP-3. RANTES and MIP-1α analogs of the present invention are preferably at least about 60% homologous to the corresponding native polypeptides and can extend to as much as 100% homology to the corresponding native polypeptides, inclusive. The RANTES analogs and MIP-1α analogs of the present invention are preferably at least about 60% homologous to the native polypeptides and more preferably at least about 75%, even more preferably at least about 85% and most preferably at least about 90% and 95% homologous to the native chemokine.

Polypeptides having truncation at both the N-terminal end and the C-terminal end are also contemplated to be within the scope of the present invention. For example, up to 10 amino acids can be

truncated from the C-terminal end of the analogs described hereinabove.

It is noted that it is well known in the art that certain amino acids can be replaced with others resulting in no or relatively little change in the properties of the polypeptide. Thus, for example, specific amino acid substitutions that may be tolerated in each analog of the present invention include: Glu or Asn for Asp; Asp or Gln for Glu; Arg for Lys; Lys for Arg; Asn for His; Pro for Gly; Gly or Thr for Pro; Asn or Met or Leu for Gln; Gln, Ser or Ala for Asn; Ser, Val or Ile or Pro for Thr; Thr or Ala for Ser; Phe for Tyr; Tyr for Phe; Ile, Val, or Met for Leu; Ser for Ala; and any combinations thereof. As defined by the present invention, e.g. "P9T" denotes a substitution of the amino acid Proline for the amino acid Threonine at position 9 of the native molecule. Similar modifications denoted by the single amino acid code followed by a molecule position number and the substituting amino acid are contemplated by the present invention (see Figure 9).

The present inventors have found that there are certain portions of the polypeptides of the present invention that must be maintained and cannot be modified. More specifically, deletion of certain amino acids from the native chemokines and the antagonists of the present invention will result in a loss of receptor binding capability. For example, deleting the disulfide bridge in MCP-3 or MCP-3 analogs (i.e. the cysteines at positions 12 and 52) will result in conformational changes and will

preclude receptor binding. Similar results have been discovered in RANTES (i.e. deletion of cysteine 11 and 50) and MIP-1α (i.e. deletion of cysteine 12 and 51).

The inventors have also discovered that extensive substitutions may be made to the C-terminal region of e.g. MCP-3 of residues 23-76 without significant loss of MCP-3 receptor binding. However, it is preferred that amino acids 13-22 of an MCP-3 analog are the same as in the native MCP-3.

The analogs are synthesized using art recognized techniques in peptide chemistry. For example, they may be synthesized by adding one amino acid at a time to an amino acid or peptide. The amino acids or peptide contain the appropriate protecting groups on the side chains and on the N-terminal portions thereof. The first amino acid containing the appropriate protecting group on the N-terminus and the side chain is coupled to a second amino acid having a protecting group on the side chain and the C-terminal end in the presence of a peptide coupling reagent, such as DCC to form the resulting peptide. Preferably, the C-terminal end is bound to a resin so that the peptide is built from the C-terminal end to the N-terminal end thereof. After the peptide is formed, another Nα-protected amino acid having side chain protection is coupled to the peptide formed previously. This process is continued until the desired peptide is formed; then the protecting groups are removed by art recognized techniques and the peptide is removed from the resin by art-recognized techniques.

An exemplary procedure for polypeptide synthesis of the compounds of the present invention is as follows. The analogs are preferably synthesized using tBoc chemistry on a peptide synthesizer and purified using reverse phase HPLC. The synthesis is started with a protected C-terminal amino acid linked to a cross-linked polystyrene resin via a 4-(carboxamidomethyl) benzyl ester linkage (the so-called pam resin) (0.4 mmol of 0.8 mmol/g of aminoacyl resin). Nα-t-Boc acids with appropriate side chain protecting groups are added in a stepwise fashion until the entire protected polypeptide chain is formed. The groups utilized for side chain protection are those commonly used in this art. Examples include: benzyl (Asp, Gly, Ser and Thr); 4-methylbenzyl (Cys); toluenesulfenyl (Arg); 2-chlorobenzyloxycarbonyl (Lys); 2-bromobenzyloxycarbonyl (Tyr); formyl (Trp); benzyloxymethyl (His); and none (Ala, Asn, Gly, Gln, Ile, Leu, Met, Phe, Pro, Val). Samples may be taken after each step to retrospectively monitor the amino acid coupling yields using a ninhydrin-based reaction following the procedures of Sarin, et al. (1981) Anal. Biochem. 117:147-157. The resin is dried and cleaved using the "low-high" hydrogen fluoride method as described by Tam, et al. (1984) J. Am. Chem. Soc. 105:6442-6485 (the contents of which are incorporated herein by reference), except for the following modifications: after the 25% hydrogen fluoride step, the partially protected peptide resin is filtered from the reaction mixture by using an all-Teflon filtration

apparatus fitted with a Zitex filter and washed with dichloromethane and dried before the high 90% hydrogen fluoride step. The ethyl acetate precipitate of the material released from the resin is dissolved in 50 ml of 6 M guanidine hydrochloride, 0.1 M Tris-acetate, pH 8.5, and 10% 2-mercaptoethanol. This mixture is the crude peptide product.

Alternately, histidine may be protected with p-benzyloxymethyl instead of dinitrophenyl. The p-benzyloxymethyl group is acid labile, thus eliminating the need for thiolysis of the dinitrophenyl group before and after hydrogen fluoride deprotection. Acetylation is carried out on the N$^{\alpha}$ deprotected, but otherwise fully protected peptide resin, using acetic anhydride (50%) in dimethylformamide.

The crude peptide product may be purified and folded by art recognized techniques. The following protocol is exemplary: three different C-18 silica HPLC columns may be used in the purification and analysis of the peptide, including a preparative column (22.4 x 250 mm column with a 22.4 x 100 mm guard column) packing with 12 μm, 300-A pore size packing (Dynamax, Rainin Instrument Co., Woburn, MA); a semipreparative column (10 x 250 mm) Vydac C018 column, with 5-μm particle, 300-A pore-size packing (Separations Group, Hesperia, CA); and an analytical column (4.6 x 250 mm) (Vydac) containing the same packing. The crude peptide product is first acidified to pH 4.0 with 20% acetic acid and filtered. The crude peptide product is then loaded onto the preparative column and the retained material eluted

with a 0-60% water-acetonitrile gradient in 0.1% trifluoracetic acid over 4 hours at a flow rate of 15 ml/min. A sample (25 µl) of fractions containing 225-nm UV-absorbing material is rerun on the analytical column and by comparison with the profile of the crude material, fractions containing the major peak are pooled and lyophilized.

To fold the protein, the material is reconstituted in 1 M guanidine hydrochloride and Tris-acetate, pH 8.5, in 10% dimethyl sulfoxide (DMSO) at a concentration of 0.2 mg/ml and stirred overnight in a covered beaker. MIP-1α is folded as above in the absence of DMSO. MIP-1α (10-70) and MIP-1α (11-70) are folded in 10% DMSO in water. This procedure promotes formation of the disulfide bridges by oxidation of the appropriate half-cysteines. The materials are acidified with 2 ml of acetic acid, and half is loaded onto the semipreparative column and the retained material is eluted with the same gradient as described above at a flow rate of 3 ml/min. Samples of each fraction are run on the analytical column. Fractions containing only material with the retention time of the major peak in the folded material are pooled and lyophilized.

An assay for free sulfhydryls using Ellman reagents, as described by Clark-Lewis, et al. (1988) Proc. Natl. Acad. Sci. USA 65:7897-7902, may be used to determine the extent of folding. In addition, folding may be monitored on the analytical HPLC column by observing the appearance of a peak corresponding to

the folded form that has a retention time approximately 3 minutes earlier than the reduced form.

Analog purity may be assessed on an analytical HPLC column or by other means such as isoelectric focusing. An exemplary protocol for isoelectric focusing is as follows. Mini polyacrylamide gels (Pharmacia PHAST gels, IEF 3-9; Pharmacia, Uppsala, Sweden) are washed in 8 M urea and then in 8 M urea containing pH 9-11 Ampholytes (Pharmacia), for 30 minutes each, either with or without 10 μM dithiothreitol. Gels are prerun for 15 V-h at 200-V, 2.0-mA, 3.0-mW maximum settings, and the samples are loaded and run for 410 V-h at 1000-V, 5.0-mA, 3.0-mW maximum settings on the Pharmacia PHAST systems for a total of 500-V with maximum settings of 2.0-mW, 5.0-mA and 1000-V. The pH gradient may be determined by using a surface pH electrode. The gels may be stained with silver by using the PHAST developing systems as described in the Pharmacia manual.

Sequencing of analogs may be determined by protein sequencing, for example by using the following protocol. Protein sequences are determined by Edman degradations using either solid-phase or gas-liquid-phase methods. For solid-phase sequence analysis, reduced and carboxymethylated protein or proteolytic cleavage fragments are coupled to arylamine-functionalized poly(vinylidenedifluoride) membranes (Sequelon AA; Milligen/Biosearch, Burlington, MA) using the water-soluble carbodiimide 1-ethyl-3-3[3-(dimethylamino)propyl]carbodiimide hydrochloride and

sequenced in a Milligen/Biosearch Model 6600 sequencer using standard protocols. For gas-liquid-phase sequence analysis, polypeptides may be applied to Polybrene-coated glass fibre disks and sequenced in an Applied Biosystems Model 477 protein sequencer using standard protocols. Sequencing of protected peptide resins may be carried out on Nα-deprotected samples by using the same methods. N-terminal solid-phase sequencing runs usually reveal a major portion of the sequence. The remaining sequence is obtained by runs of the HPLC-fractionated fragments, derived either by proteolytic cleavage with Asp-N-endoprotease (Boehringer Mannheim Canada, Laval, Quebec) or by chemical cleavage, through preferential hydrolysis of the Asp-Pro peptide bond in dilute formic acid.

Molecular weight of the synthetic proteins prepared as described above are determined by art-recognized techniques, such as electrospray mass spectrometry on a SCIEX triple quadruple Mass Spectrometer equipped with a liquid delivery apparatus. The molecular mass from the peaks corresponding to the charge to mass ratios of the different multiple ionized species of the protein can also be analyzed as described by Convey, et al. (1988) Rapid Commun. Mass. Spectrom. 2:249-256.

The chemokine analogs of this invention may also be prepared through recombinant means, with expression in mammalian or non-mammalian systems. Portions of a DNA sequence encoding, e.g., MCP-3, are appropriately modified to produce the desired analog when the DNA sequence is expressed. Methods and

protocols for preparation and expression of such recombinant DNA are known in the art, including the protocol described by Masure, et al. (1995) J. Interferon Cytokine Res. 15:955-963 used for production of mutant MCP-3 proteins, incorporated herein by reference.

In accordance with the present invention, the inventors have discovered that, in contrast to the full-length native forms, the analogs of the present invention were antagonists to the corresponding native molecules. Moreover, they lacked detectable chemoattractant activity for the receptor carrying cells. For example, RANTES (9-68), MCP-3 (10-76) and MIP-1α (10-70) lacked detectable chemoattractant activity for human THP-1 monocytic cells. RANTES (6-68), (7-68), (8-68) and (10-68) also lacked chemoattractant activity for T-cells, monocytes, NK cells, basophils and eosinophils. MCP-3 (8-76), (9-76) and (11-76) also lacked activity for human CD4+ and CD8+ T lymphocytes, NK cells, eosinophils and basophils. MIP-1α similarly lacked chemoattractant activity for monocytes and human T-cells.

In accordance with the present invention, the inventors have observed certain regions within the native chemokines which are essential for their functional activities. Specifically, NH_2-terminal amino acid residues within the 1-5 region of native RANTES, residues within the 1-7 region of native MCP-3 and residues within the 1-9 region of native MIP-1α are critical for chemoattractant activity and receptor activation, for example. However, by truncating the

1-5 region of native RANTES, 1-7 region of native MCP-3 and 1-9 region of native MIP-1α, the resulting molecules become antagonists to the native chemokines.

The inventors have also discovered that the truncated chemokine analogs of the present invention act as antagonists to native, full-length chemokines and do not possess the chemoattractant activity. For example, RANTES(9-68), MCP-3(10-76) and MIP-1α (10-70) inhibited the biological activity (e.g. chemoattractant activity and N-acetyl-β-D-glucosaminidase release, elastase release and intracellular calcium mobilization) induced by the corresponding native forms.

In accordance with the present invention, the inventors have assessed the receptor-binding specificity of the truncated analogs. The inventors have discovered significant cross-receptor binding among the identified chemokine analogs of the present invention. Thus, for example, unexpectedly, RANTES (9-68) inhibited native RANTES, MCP-3, MCP-1 and MIP-1α-induced chemotaxis and N-acetyl-β-D-glucosaminidase release and intracellular calcium mobilization.

The inventors also observed that MCP-3 (9-76) and MCP-3 (10-76) inhibited native MCP-3-induced, RANTES-induced, MCP-1-induced, calcium mobilization, monocyte N-acetyl-β-D-glucosaminidase release and chemoattractant activity.

The inventors have further observed that MIP-1α (10-70) inhibited native MIP-1α/β-induced and RANTES-induced monocyte N-acetyl-β-D-glucosaminidase

release and chemoattractant activity and calcium mobilization.

The present inventors have determined that the polypeptides (analogs) of the present invention act as antagonists to the effects of native RANTES, MIP-1α and MCP-3 in various tests, such as chemotaxis, calcium mobilization, receptor binding in THP-1 cells (a monocytic cell line). Based upon these observations, the inventors concluded that the polypeptides of the present invention are useful in blocking the effects of native RANTES, MIP-1α and MCP-3 molecules and thus are useful as therapeutic agents. A preferred use of the polypeptides of the present invention is in blocking the effects of RANTES, MIP-1α and MCP-3 in the recruitment and/or activation of pro-inflammatory cells. Thus, the present invention has utility in the treatment of such inflammatory diseases as asthma, allergic rhinitis, colitis, psoriasis, atheromas, dermatitis and the like. In addition, the polypeptides are also useful in the treatment of autoimmune diseases, e.g., rheumatoid arthritis. For example, the inventors have observed that the analogs of the present invention (e.g. RANTES antagonists) are useful in treating autoimmune disorders (e.g., rheumatoid arthritis) in mammals. The inventors have determined that the analogs of the present invention prevent the onset of rheumatoid arthritis in MRL-1pr mice. The inventors have discovered that administration of e.g., RANTES(9-68) significantly inhibited the clinical incidence of adjuvant enhanced arthritis for 30 days in an autoimmune mouse model

(MRL-1pr mice have lupus erythematous and rheumatoid arthritis similar to the corresponding human diseases).

In another aspect of the present invention, methods for inhibiting the biological activity of native chemokines are provided which involve administering a therapeutically effective amount of a truncated analog of the present invention to a mammal to treat, e.g. inflammatory diseases and autoimmune diseases.

In another aspect of the present invention, the NH_2-terminally truncated chemokine analogs of the present invention are administered in pharmaceutical compositions. The analogs of this invention may be administered as a nasal spray for upper respiratory treatments or as an aerosol inhaler for lung conditions. The analogs may also be used in topical applications. The analogs may also be administered via an osmotic pump or in a sustained release formulation. Alternatively, the analogs of the present invention may be delivered by injection. It is preferred that the injection is intramuscular or intraanal. However, the present invention also contemplates intradermal, intraperitoneal or intraarticular injections as well as long term continuous delivery via subcutaneous osmotic pump or sustained release formulations.

Other methods of in vivo and in vitro use of the chemokine analogs of the present invention will be readily apparent from the examples herein and the assays described above. The chemokine analogs of the

present invention are present in the various pharmaceutical formulations described hereinabove in an "antagonistically effective amount" or "therapeutically effective" amount. "Antagonistically effective amount" or "therapeutically effective" amount as used herein is defined as an amount of chemokine analog sufficient to significantly inhibit the biological activity of native chemokines but low enough to avoid serious side effects such as toxicity, for example (at a reasonable risk/benefit ratio) within the sound medical/scientific judgment of the skilled artisan. However, it is preferred that the dose of the formulation contains between 0.01-1 mg/kg for a nasal spray and 0.1-10 mg/kg for other forms of delivery. It is most preferred that the formulation contains between 0.01-0.1 mg/kg for a nasal spray and 0.1-1 mg/kg for other forms of delivery. The dosage schedule of the chemokine analogs will typically be determined (at a reasonable risk/benefit ratio) within the sound medical/scientific judgement of the skilled artisan. However, it is preferred that the chemokine analogs are administered at least about 3 times per week. It is most preferred that the chemokine analogs are administered on a daily basis.

The exact dose of a chemokine analog of the present invention to be used in a particular application may be determined by accepted pharmaceutical methods known to the skilled artisan. This is accomplished by conventionally measuring the concentration of analog in the blood and determining the analog half-life. Without wishing to be bound, it

is believed that the analogs of the present invention have what would otherwise be an unexpectedly high half life for similar polypeptides due to molecular stability at body temperature and due to the high binding affinity to their receptors.

Pharmaceutical compositions comprising the analogs of the present invention additionally contain pharmaceutical vehicles, such as carriers and adjuvants described in the literature of pharmaceuticals and related fields. The analogs of the present invention are formulated by the skilled artisan, using art-recognized techniques, taking into consideration the nature of the polypeptide compounds and the desired mode of administration. The product of the present invention are soluble and are therefore readily formulated in physiological buffers, e.g. physiological saline.

The inventors have observed that unlike other chemokines, native human RANTES and MIP-1α form aggregates in physiological conditions (e.g. at about pH 7.2) at concentrations as low as 100 nM. This presents a significant hurdle to in vivo applications because therapeutic efficacy usually requires much higher concentrations of the active compounds (i.e., up to 2 mM). However, the present inventors have surprisingly discovered that the truncated chemokine analogs of the present invention exhibit no aggregation at concentrations above about 1 μM. Therefore, the truncated chemokine analogs (e.g. RANTES and MIP-1α) of the present invention are highly

soluble, and thus facilitate more in vivo applications than their native counterparts.

In addition, the chemokine analogs of the present invention can also formulated in sustained release delivery systems or topical formulations containing an aqueous component.

MCP-3, RANTES and MIP-1α analogs of the present invention are assayed for biological activity by use of a cytosolic-free calcium assay, a chemotaxis assay using cells of monocytic origin, or by other conventional assays for MCP-3, RANTES or MIP-1α activity including but not limited to: assays to determine exocytosis of leukocytes such as elastase release and N-acetyl-β-D-glucosaminidase release, superoxide production, histamine release, LTC_4 release and the like.

The invention will now be illustrated by means of the following non-limiting examples.

In the following example, analysis of cytosolic-free calcium was carried out using the following protocol. Cells (4×10^5) are loaded with 12, 5 µg/ml Fluo-3AM or Fluo-2 in PBS saline with 0.38 mg/ml Pluronic F127 (Molecular Probes, Eugene, OR) at 37°C for 30 minutes. After washing with PBS, the cells were resuspended in 25 mM Hepes, 140 mM NaCl, 10 mM glucose, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, and 3 mM KCl, pH 7.3. The fluorescence was monitored at 7 second intervals over 150 seconds, after addition of test sample. Maximum Ca^{2+} levels were established using Fluo-3AM or Fluo-2 (Designated 100% saturation) for

each set of measurements by addition of 5 μM Ionomycin (Sigma Chemical Co., St. Louis, MO).

EXAMPLE 1

Representative polypeptides of the present invention were tested for their functional activity according to the procedure described hereinabove. The results are as follows. In contrast to the full-length native forms, RANTES (9-68), MCP-3 (10-76) and MIP-1α (10-70) lacked detectable chemoattractant activity for human THP-1 monocytic cells (Figures 1A-1C). In addition, for human blood-derived monocytes, neither chemotaxis nor N-acetyl-β-D-glucosaminidase release was detected up to 1 μM. RANTES (6-68), (7-68), (8-68) and (10-68) as well as MCP-3 (8-76), (9-76) and (11-76) also lacked chemoattractant activity (Figure 1B). MIP-1α (10-70) had only minor activity (about 3% of that of the corresponding native MIP-1α). MIP-1α (11-70) had no detectable activity. Thus, residues within the 1-5 region of native RANTES, 1-7 region of native MCP-3 and 1-9 region of native MIP-1α are essential for the functional activities of the native chemokines. Truncation of these regions resulted in a complete loss of e.g. chemoattractant activity of the native chemokines.

EXAMPLE 2

Inhibition of native MCP-3, RANTES or MIP-1α mediated chemotaxis are determined by using the aforementioned chemotaxis assay. Constant amounts of MCP-3, RANTES or MIP-1α (e.g. 10 nM) are added to each well, and the analogs are titrated in the assay.

Cell preparations for use in the aforementioned assays consist of human monocytes, or monocytic cell lines such as the cell line THP-1. THP-1 were obtained from American Type Culture Collection (Rockville, MD) and may be, optionally, maintained in RPMI 1640 medium supplemented with 10% FCS.

Human monocytes were isolated from buffy coats of normal donor blood by the following protocol. A cell suspension was loaded onto Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) and centrifugated at 400 g for 25 minutes followed by density centrifugation on a discontinuous Percoll (Pharmacia) gradient at 500 g for 30 minutes. Cell fractions with a density of 1.051-1.053 (g/ml) were generally greater than 70% monocytes by morphology and were used in the assay.

RANTES (9-68), MCP-3 (10-76), MIP-1α (10-70) and MIP-1α (11-70) were tested for their antagonist activities. All three inhibited the activity induced by the corresponding native forms (Figures 2A-2D). RANTES (9-68) fully blocked the chemoattractant activity of RANTES for THP-1 cells, and 41 nM was required to inhibit the agonist activity of 10 nM native RANTES by 50% (IC_{50} = 41 nM). Similar results

were obtained for migration of peripheral blood monocytes. The ability of RANTES (9-68) to inhibit chemokine-induced exocytosis from human blood monocytes was assessed by measuring the release of N-acetyl-β-D-glucosaminidase. A 10-fold lower concentration (IC_{50} = 4 nM) of RANTES (9-68) was required to inhibit RANTES-induced enzyme release than was needed for similar inhibition of chemotaxis (Figure 3A).

To test the specificity of the three truncated antagonists, their ability to inhibit the activities induced by RANTES, MCP-3 and MIP-1α was examined (Figures 3A-3D). The RANTES (9-68) analog completely inhibited both MCP-3 and MIP-1α induced chemotaxis and N-acetyl-β-D-glucosaminidase release. The respective IC_{50} values were 200 nM and 126 nM for THP-1 cell chemotaxis and 170 nM and 20 nM for monocyte release activity (Figure 6). Thus RANTES (9-68) inhibited all the chemokines, but it had the highest potency for RANTES.

MCP-3 (10-76) was found to inhibit MCP-3 induced monocyte N-acetyl-β-D-glucosaminidase release with an IC_{50} of 37 nM and also chemoattractant activity IC_{50} 470 nM. Thus it was less potent than RANTES (9-68) for RANTES-stimulated activities. However, MCP-3 (10-76) also inhibited RANTES-induced and MIP-1α-induced activities with similar effectiveness to its inhibition of MCP-3 elicited function (Figure 3B). Furthermore, MCP-3 (10-76) blocked enzyme release from monocytes. The IC_{50} values for inhibiting both RANTES or MCP-3 induced release were around 10-fold lower

than for chemotaxis of either THP-1 cells or monocytes. Another MCP-3 variant, MCP-3 (9-76), was 2-3 fold more potent than MCP-3 (10-76) in all the inhibition assays.

MIP-1α (10-70) was found to inhibit MIP-1α or RANTES induced monocyte-N-acetyl-β-D-glucosaminidase release with an IC_{50} of 1 nM (Figures 3C-3D). MIP-1α (10-70) was also found to inhibit monocyte/THP-1 cell migration. MIP-1α (11-70) also inhibited both chemoattractant activity and enzyme release of native MIP-1α and native RANTES with an IC_{50} of 30 nM and 10 nM, respectively. Because MIP-1α antagonists did not inhibit MCP-3 activity, MIP-1α antagonists are relatively selective for MIP-1α and RANTES. Antagonists of native RANTES and native MCP-3 have relatively broad inhibitory spectra.

EXAMPLE 3

MCP-3, RANTES or MIP-1α receptor binding are determined by the following protocol. MCP-3 (10 µg) were labeled with monoiodinated Bolton-Hunter reagent (specific activity 2,200 Ci/mmol; DuPont, Wilmington, DB) at 4°C for 20 minutes, to provide specific activity of, e.g. ^{125}I-labeled MCP-3 of 150 Ci/mmol. RANTES and MIP-1α were labeled using lactoperoxidase. One mCi (3.7 MBq) of Na^{125} (ICN Biomedicals, Irvine CA) and 1 µg of lactoperoxidase (80-150 units-mg^{-1}, Sigma) were added to 5 µg of RANTES or MIP-1α in 50 µl of 0.5 M sodium acetate, pH 6.5 at room temperature for 3 min. Saturated tyrosine (150 µl) was added to stop the reaction, and the proteins were separated from the label by Sephadex G-25 chromatography. The specific activity of ^{125}I-labeled RANTES was 260 Ci-mmol^{-1}.

To determine the binding kinetics, monocytic cells(such as THP-1) at (5 x 10^6 cells) in 200 µl of binding buffer (RPMI 1640, 0.5 mg/ml BSA, 50 mM Hepes and 0.01% NaI) were incubated with varying concentrations of ^{125}I-MCP-3 at 4°C for 30 minutes. The cells were pelleted through a mixture of diacetylphthalate and dibutylphthalate and radioactivity that is cell associated was counted (total binding). Nonspecific binding was determined in the presence of a 100-fold concentration of unlabeled ligand and subtracted from the total binding. Kinetic parameters (Kd and receptor number) were determined by Scatchard analysis.

Competitive receptor binding by, e.g. MCP-3, analogs is measured by carrying out the aforementioned receptor binding protocol wherein various concentrations of e.g. MCP-3 analogs are added to the cells in the presence of e.g., 4 nM ^{125}I-MCP-3. Non-specific binding is subtracted from total binding and the result is expressed as a percent of maximum specific binding (See Figures 4A-4E and Figure 5).

Competition binding studies were performed to determine if the observed antagonist properties correlated with their interaction with chemokine binding sites.

The RANTES (9-68) analog competed for binding of labeled RANTES. From this data, the dissociation constant (K_d) was calculated to be 19 nM, that is only about 4-5 fold of that of native RANTES binding affinity (4 nM). Furthermore, RANTES (9-68) also displaced labeled MCP-3 (K_d = 57 nM). Although, as expected, native RANTES competed strongly for RANTES binding, its competition for labeled MCP-3 was very weak and insufficient to derive a K_d value. Thus, truncation of RANTES resulted in a markedly increased affinity for MCP-3 binding sites.

MCP-3 (10-76) competed for MCP-3 binding with a K_d of 57 nM and also competed for binding of both RANTES (K_d = 50 nM) and MIP-1α. The competition of the MCP-3 (10-76) analog for labeled MCP-3 was only 2-fold weaker than that of full-length MCP-3. For RANTES receptors, MCP-3 and MCP-3 (10-76) had about the same affinity. The results indicate that MCP-3 (10-76) had similar affinity for the binding sites of

all three chemokines. Similar receptor binding affinity data were obtained with MCP-3 (9-76). MCP-3 (8-76); MCP-3 (7-76) and MCP-3 (6-76) had lower receptor binding affinities than MCP-3 (9-76) and MCP-3 (10-76).

The MIP-1α (10-70) analog competed for binding of labeled MIP-1α with a Kd of 25 nM and also competed for binding of RANTES (Kd = 14 nM) and less for MCP-3 (Kd = 281 nM). Native MIP-1α and MCP-1α (10-70) had about the same affinity for RANTES receptors. MIP-1α (11-70) competed only for RANTES and MIP-1α receptor binding. No competition for MCP-3 was observed. Therefore MIP-1α (10-70) has higher inhibitory potency than MIP-1α (11-70).

A further assay that may be carried out to determine whether a non-chemotactic analog binds to, e.g. MCP-3 receptors is to measure the ability of an analog to desensitize calcium mobilization by MCP-3. Following a first treatment with a MCP-3 receptor ligand, the calcium response is temporally desensitized to a second treatment with a MCP-3 receptor ligand. This is determined by carrying out the aforementioned cytosolic-free calcium assay with addition of a first ligand, followed by a second treatment after 60 seconds using either the same of a different ligand. A MCP-3 antagonist will not of itself stimulate calcium induction but when used as the first ligand, will desensitize the cells to subsequent stimulation by MCP-3. The following example illustrates the result.

EXAMPLE 4

The truncated analogs RANTES (9-68) and MCP-3 (10-76) were tested for their ability to inhibit the transient rise of $[Ca^{2+}]$ induced in monocytes by native RANTES and MCP-3. In all cases the truncated analogs inhibited the $[Ca^{2+}]$ rise induced by the native chemokines. The most potent effect was observed with RANTES (9-68), which at 30 nM totally prevented the $[Ca^{2+}]$ changes induced by 10 nM RANTES. For inhibition of the responses to MCP-3, markedly higher concentrations of the corresponding truncated analogs were required. At high concentration (1,000 nM), all truncated analogs also attenuated or even prevented the $[Ca^{2+}]$ changes induced by the other chemokines. RANTES (9-68) markedly decreased the $[Ca^{2+}]$ induced by MCP-3.

EXAMPLE 5

Three month old MRL-1pr mice, both male and female were injected at two thoracic sites with complete Freunds Adjuvant (CFA) only once on day 0. On that same day RANTES(9-68) was injected intraperitoneally. Injections of the RANTES analog were administered daily for 30 days at 2mg/kg/day. The mice were visually examined by (a double-blind observation) every five days for appearance of arthritis. An animal was scored as positive for arthritis if erythema and swelling of a fore or hind paw was observed. In addition ankle width of the hind legs were assessed. Histopathology of the lime joints were evaluated on day 30 post-adjuvant injection.

RESULTS:

Human RANTES(9-68) was able to bind to the receptor on mouse peritoneal macrophages. Native human RANTES did not bind well to mouse peritoneal macrophages. Human RANTES(9-68) bound to mouse peritoneal cells with an affinity (Kd) of 21 nM which is similar to the binding affinity of RANTES(9-68) to human monocytes (Figure 8).

Daily i.p. injections of RANTES (9-68) inhibited the swelling and the incidence of arthritis in adjuvant treated mice. Treated mice exhibited insignificant swelling. In contrast, treatment with a control peptide had no inhibitory effect (Figures 9A and 9B).

Histopathological studies demonstrated that untreated control animals and animals administered a peptide control developed rheumatoid arthritis, substantial infiltration of mononuclear cells into the synovial tissue, extensive hyperplasia of the synovial lining, pannus formation and bone and cartilage damage. In contrast, animals treated with RANTES(9-68) exhibited no invasive pannus, no bone or cartilage damage and insignificant inflammatory infiltration (Figure 10).

Treatment with human RANTES(9-68) for 30 days was not toxic.

EXAMPLE 6

Three month old MRL-1pr mice, both male and female were injected at two thoracic sites with complete Freunds Adjuvant (CFA) only once on day 0. On that same day human MCP-3 antagonist MCP-3 (9-76) was injected intraperitoneally. Injections of the MCP-3 analog were administered daily for 30 days at 3 mg/kg/day. The mice were visually examined (by a double-blind observation) every five days for appearance of arthritis. An animal was scored as positive for arthritis if erythema and swelling of a fore or hind paw was observed. In addition ankle width of the hind legs was assessed. Histopathology of the lime joints were evaluated on day 30 post-adjuvant injection.

RESULTS:

Human MCP-3 (9-76) was able to bind to the receptor on mouse monocytic cells (WEH1 265 cells). Human MCP-3 (9-76) did not bind well to mouse monocytes. Human MCP-3 (9-76) bound to mouse monocytic cells with an affinity (kd) of 57 nM which is four-fold lower than that for native MCP-3 (kd 225 nM) (Figure 11).

Daily i.p. injections of MCP-3 (9-76) inhibited the swelling and the incidence of arthritis in adjuvant treated mice. In contrast, treatment with a control peptide had no inhibiting effect (Figure

12). Treatment with human MCP-3 (9-76) for 30 days was not toxic.

Thus, in summary, in accordance with the present invention, the inventors have discovered that NH_2-terminally truncated analogs of native chemokines lack chemoattractant and other activities. The present inventors have also determined that the analogs of the present invention competitively bind to native chemokine receptors, thereby significantly inhibiting or even precluding binding by the corresponding native molecules. The inventors have also discovered a correlation between the bound analogs and their concomitant lack of biological activity. It has now been discovered by the present inventors that the analogs of the present invention are useful in treating inflammatory diseases and autoimmune diseases in mammals.

WHAT IS CLAIMED IS:

1. An analog of mammalian Monocyte Chemoattractant Protein-3 (MCP-3) lacking NH_2-terminal amino acids corresponding to amino acid residues 1-7, 1-8, 1-9 or 1-10 of MCP-3, having about at least 80% homology to said MCP-3 and having antagonist activity thereto.

2. The analog according to Claim 1, wherein said analog has at least 85% homology to MCP-3.

3. The analog according to Claim 1, wherein said analog has at least 90% homology to MCP-3.

4. The analog according to Claim 1, wherein said analog has at least 95% homology to MCP-1.

5. The analog according to Claim 1, wherein said analog comprises MCP-3 (9-76) or a fragment thereof.

6. An analog of mammalian Regulated on Activation Normal T Cell Expressed and Secreted protein (RANTES) lacking NH_2-terminal amino acids corresponding to amino acid residues 1-5, 1-6, 1-7, 1-8, and 1-9 of RANTES, having about at least 60% homology to said RANTES and having antagonist activity to said RANTES.

7. The analog according to Claim 6, wherein said analog comprises RANTES (9-68) or a fragment thereof.

8. The analog according to Claim 6, wherein said analog comprises RANTES (6-68) or a fragment thereof.

9. The analog according to Claim 6, wherein said analog comprises RANTES (7-68) or a fragment thereof.

10. The analog according to Claim 6, wherein said analog comprises RANTES (8-68) or a fragment thereof.

11. The analog according to Claim 6, wherein said analog comprises RANTES (10-68) or a fragment thereof.

12. An analog of mammalian Macrophage Inflammatory Protein-1α (MIP-1α) lacking NH_2-terminal amino acids corresponding to amino acid residues 1-9 or 1-10, having about at least 60% homology to said MIP-1α and having antagonist activity to said MIP-1α.

13. The analog according to Claim 12, wherein said analog comprises MIP-1α (10-70) or a fragment thereof.

14. A method of inhibiting MCP-3 biological activity in a mammal comprising administering to said mammal a therapeutically effective amount of an analog according to Claim 1, 6, or 12 for a time and under conditions sufficient to inhibit said biological activity.

15. A method of inhibiting RANTES biological activity in mammals comprising administering thereto a therapeutically effective amount of an analog according to Claims 1, 6, or 12 for a time and under conditions sufficient to inhibit said biological activity.

16. A method of inhibiting MIP-1α biological activity in mammals comprising administering thereto a therapeutically effective amount of an analog according to Claims 1, 6, or 12 for a time and under conditions sufficient to inhibit said biological activity.

17. A method of treating an inflammatory disease in a mammal comprising administering to a mammal in need thereof a therapeutically effective amount of an analog according to Claims 1, 6 or 12.

18. A method of treating an autoimmune disorder in a mammal comprising administering to a mammal in need thereof a therapeutically effective amount of an analog according to Claims 1, 6 or 12.

19. A method of treating rheumatoid arthritis in a mammal comprising administering to a mammal in need thereof a therapeutically effective amount of an analog according to Claim 1, 6 or 12.

20. A pharmaceutical composition comprising an antagonistically effective amount of an analog of Claim 1 and a pharmaceutically acceptable carrier.

21. A pharmaceutical composition comprising an antagonistically effective amount of an analog of Claim 6 and a pharmaceutically acceptable carrier.

22. A pharmaceutical composition comprising an antagonistically effective amount of an analog of Claim 12 and a pharmaceutically acceptable carrier.

FIG. 1A

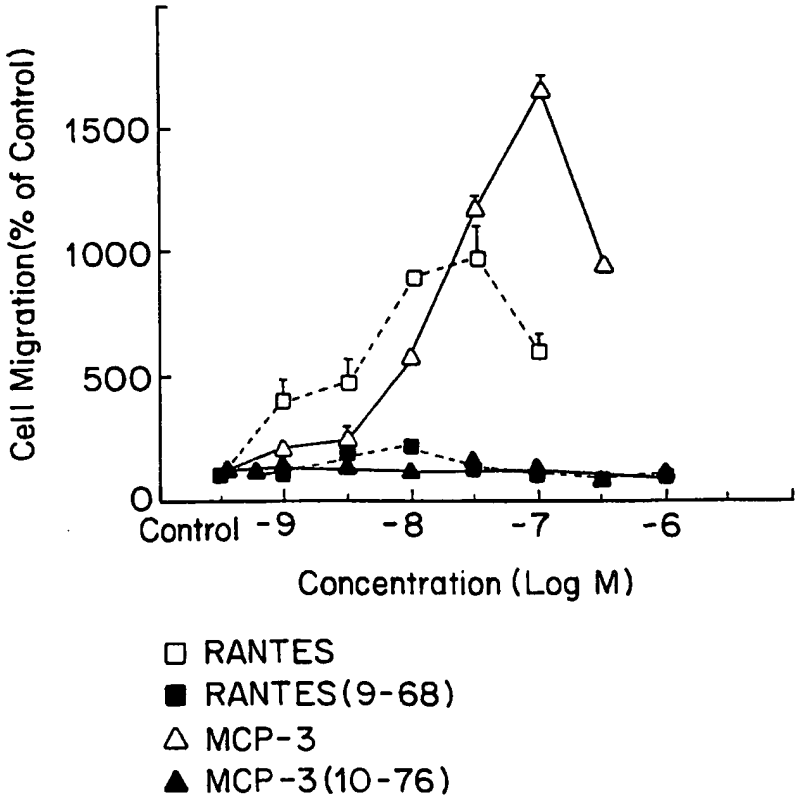

Cell Migration(% of Control)
1500
1000
500
0
Control
-9
-8
-7
-6
Concentration (Log M)
RANTES
RANTES(9-68)
MCP-3
MCP-3(10-76)

FIG. 1B

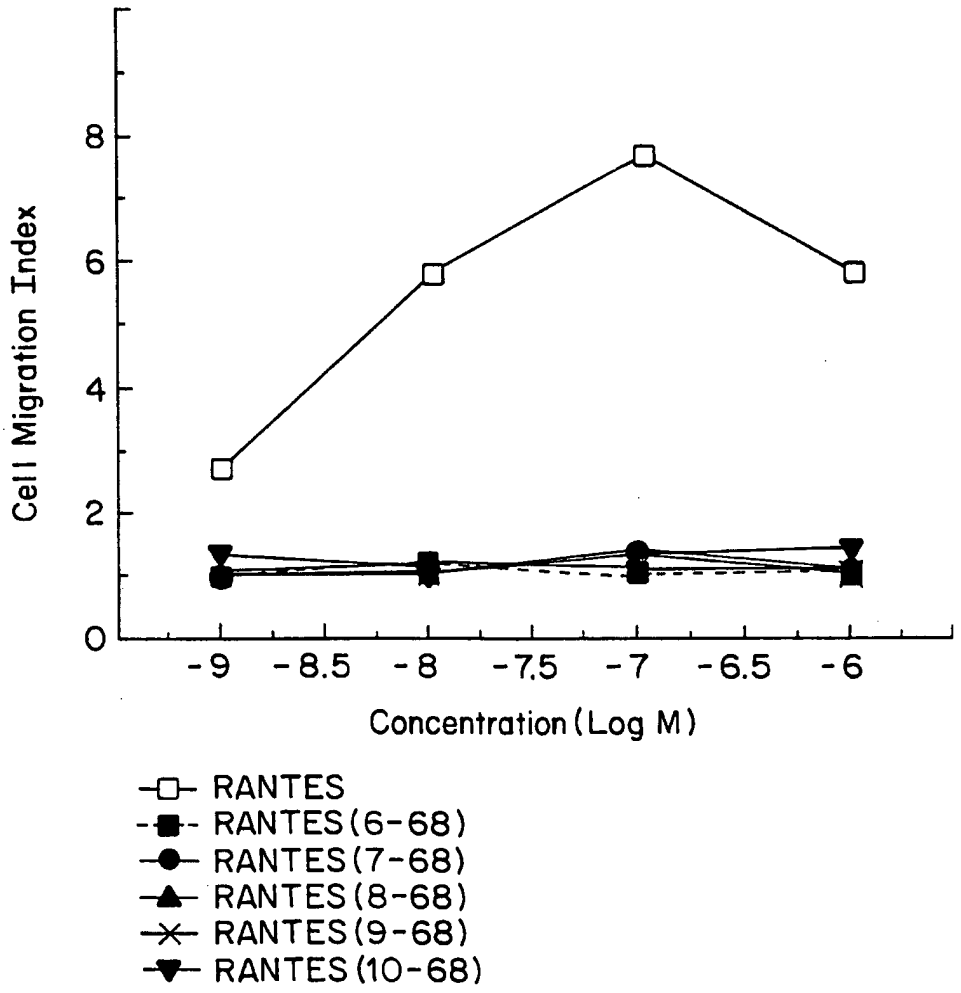

Cell Migration Index
0
2
4
6
8
-9
-8.5
-8
-7.5
-7
-6.5
-6
Concentration (Log M)
RANTES
RANTES (6-68)
RANTES (7-68)
RANTES (8-68)
RANTES (9-68)
RANTES (10-68)

FIG. IC

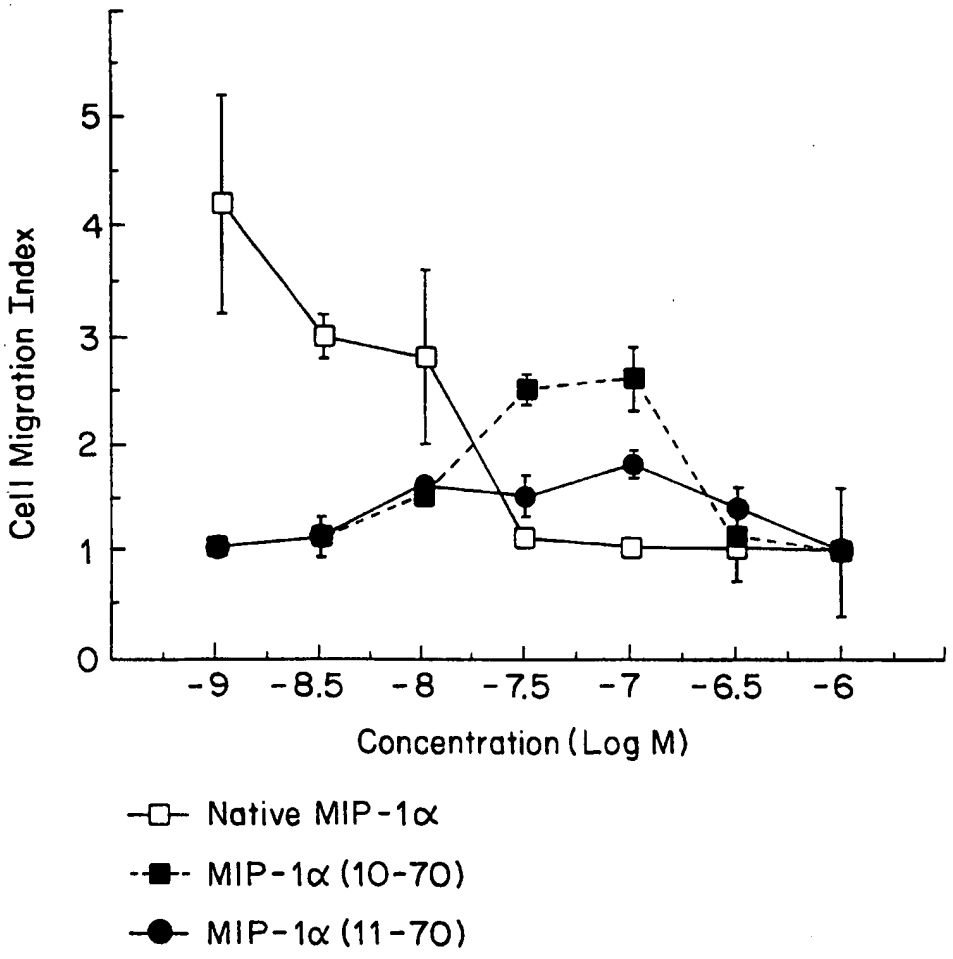

Cell Migration Index
0
1
2
3
4
5
-9
-8.5
-8
-7.5
-7
-6.5
-6
Concentration (Log M)
Native MIP-1α
MIP-1α (10-70)
MIP-1α (11-70)

FIG. 2A

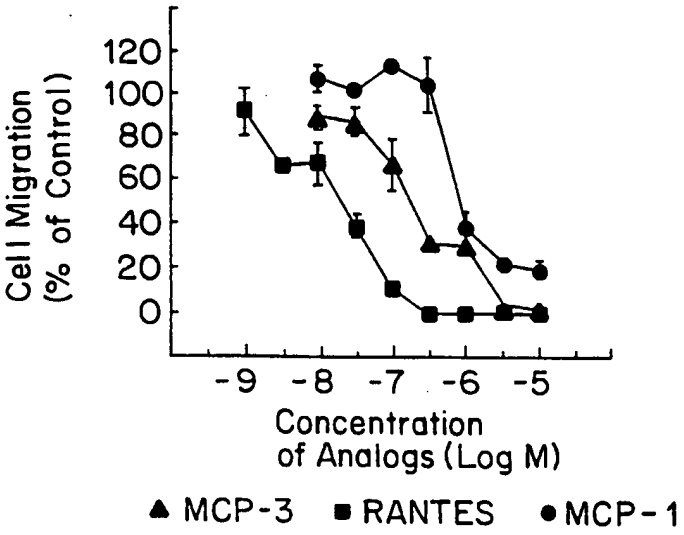

Cell Migration
(% of Control)
120
100
80
60
40
20
0
-9 -8 -7 -6 -5
Concentration
of Analogs (Log M)
▲ MCP-3 ■ RANTES ● MCP-1

FIG. 2B

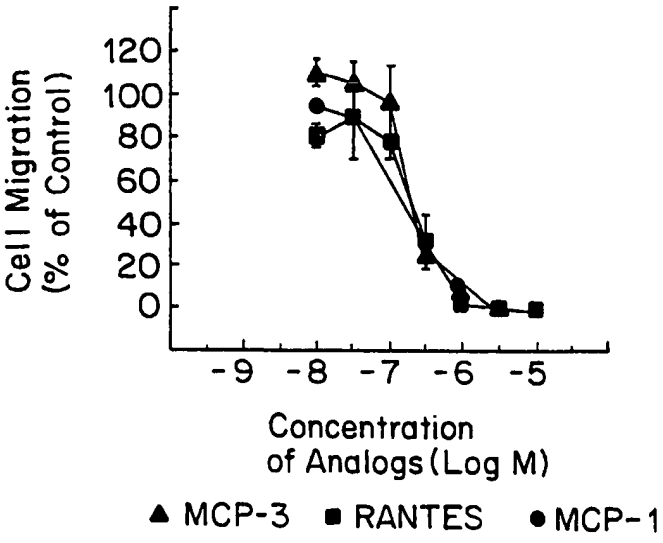

Cell Migration
(% of Control)
120
100
80
60
40
20
0
-9 -8 -7 -6 -5
Concentration
of Analogs (Log M)
▲ MCP-3 ■ RANTES ● MCP-1

FIG. 2C

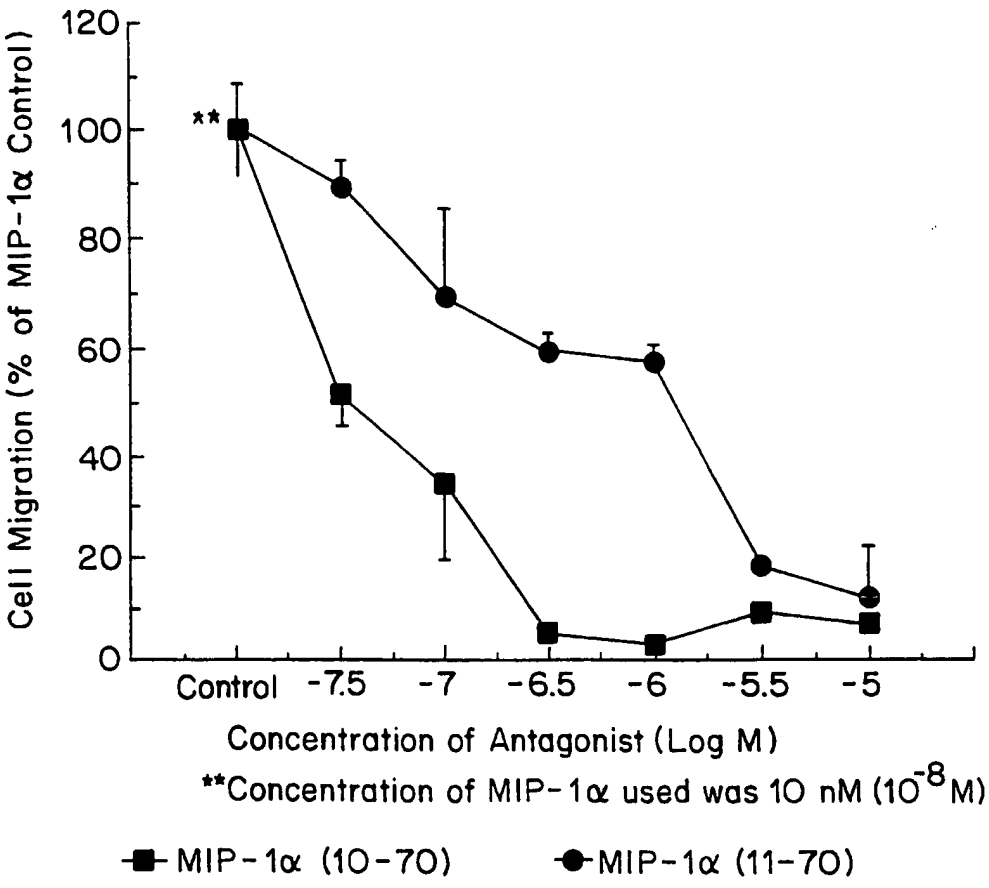

120
100
80
60
40
20
0
Cell Migration (% of MIP-1α Control)
**
Control
-7.5
-7
-6.5
-6
-5.5
-5
Concentration of Antagonist (Log M)
**Concentration of MIP-1α used was 10 nM (10^{-8} M)
MIP-1α (10-70)
MIP-1α (11-70)

FIG. 2D

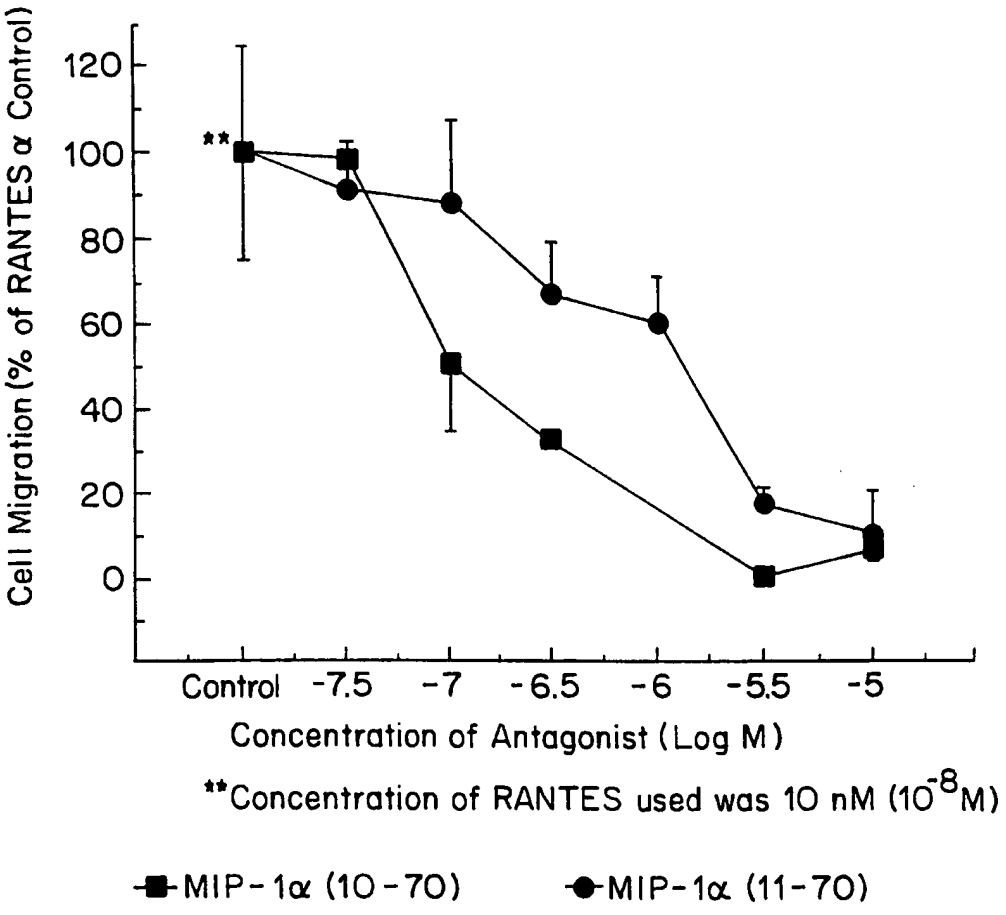

Cell Migration (% of RANTES α Control)
120
100
80
60
40
20
0
**
Control
-7.5
-7
-6.5
-6
-5.5
-5
Concentration of Antagonist (Log M)
**Concentration of RANTES used was 10 nM (10^{-8}M)
MIP-1α (10-70)
MIP-1α (11-70)

FIG. 3A

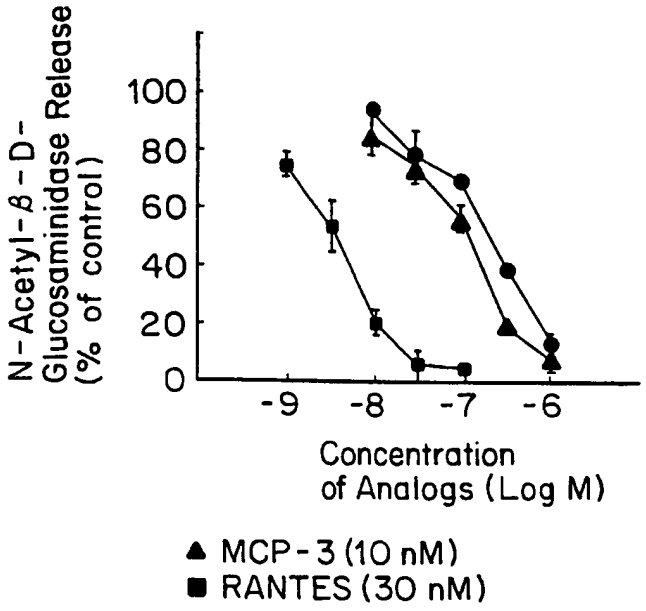

N-Acetyl-β-D-Glucosaminidase Release (% of control)
100
80
60
40
20
0
-9
-8
-7
-6
Concentration of Analogs (Log M)
▲ MCP-3 (10 nM)
■ RANTES (30 nM)

FIG. 3B

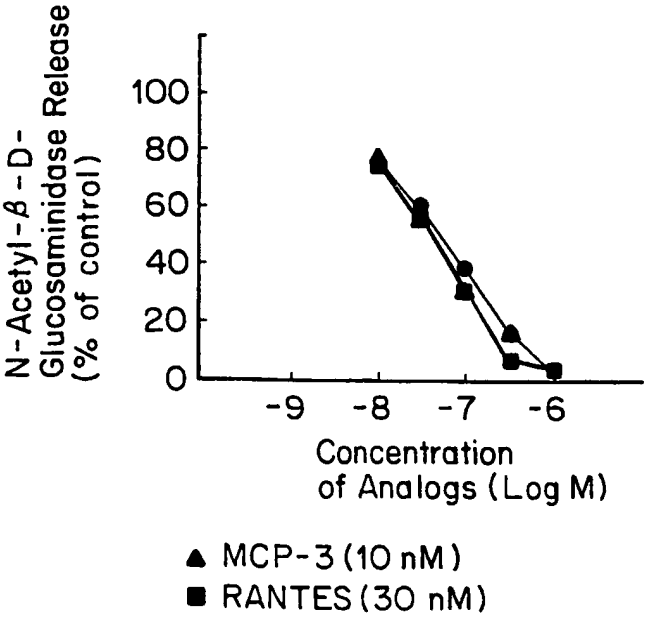

N-Acetyl-β-D-Glucosaminidase Release (% of control)
100
80
60
40
20
0
-9
-8
-7
-6
Concentration of Analogs (Log M)
▲ MCP-3 (10 nM)
■ RANTES (30 nM)

FIG. 3C

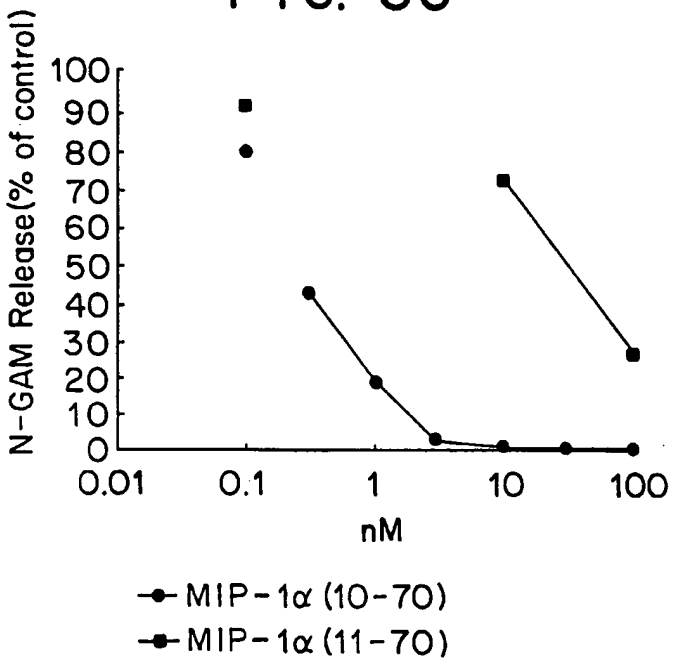

N-GAM Release(% of control)
100
90
80
70
60
50
40
30
20
10
0
0.01
0.1
1
10
100
nM
MIP-1α (10-70)
MIP-1α (11-70)

FIG. 3D

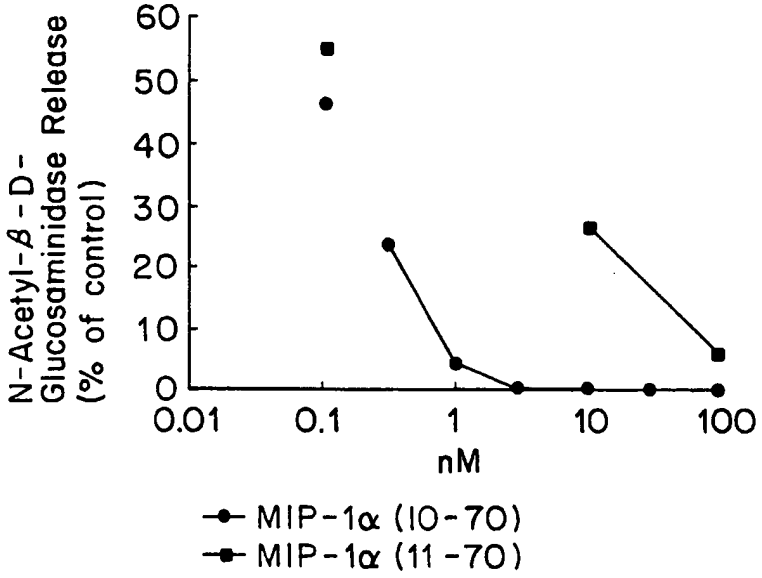

N-Acetyl-β-D-Glucosaminidase Release (% of control)
60
50
40
30
20
10
0
0.01
0.1
1
10
100
nM
MIP-1α (10-70)
MIP-1α (11-70)

FIG. 4A

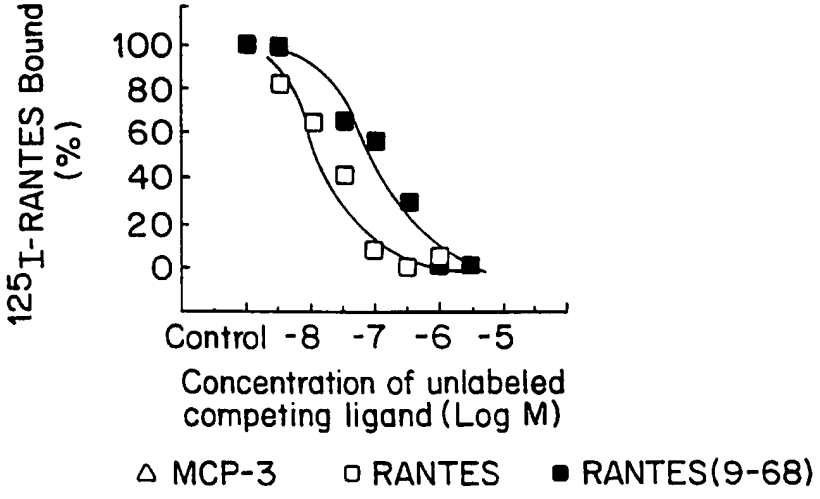

125I-RANTES Bound (%)
100
80
60
40
20
0
Control -8 -7 -6 -5
Concentration of unlabeled competing ligand (Log M)
△ MCP-3 □ RANTES ■ RANTES(9-68)

FIG. 4B

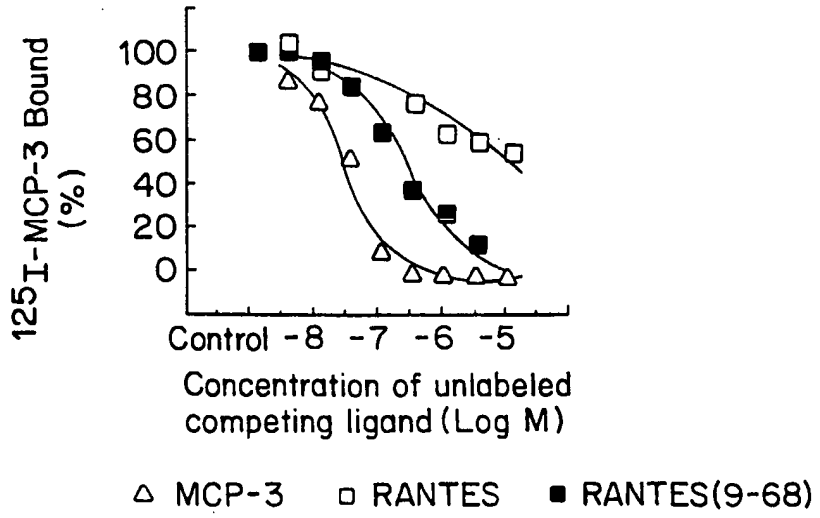

125I-MCP-3 Bound (%)
100
80
60
40
20
0
Control -8 -7 -6 -5
Concentration of unlabeled competing ligand (Log M)
△ MCP-3 □ RANTES ■ RANTES(9-68)

FIG. 4C

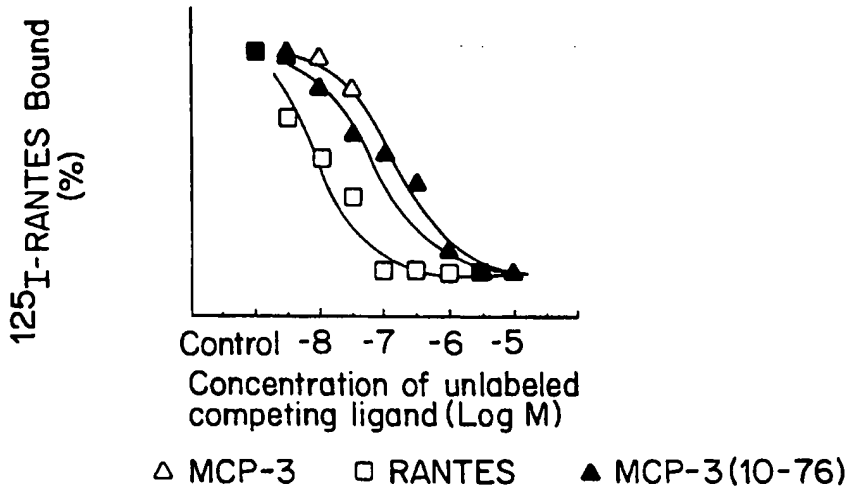

^{125}I-RANTES Bound (%)
Control -8 -7 -6 -5
Concentration of unlabeled competing ligand (Log M)
△ MCP-3 □ RANTES ▲ MCP-3(10-76)

FIG. 4D

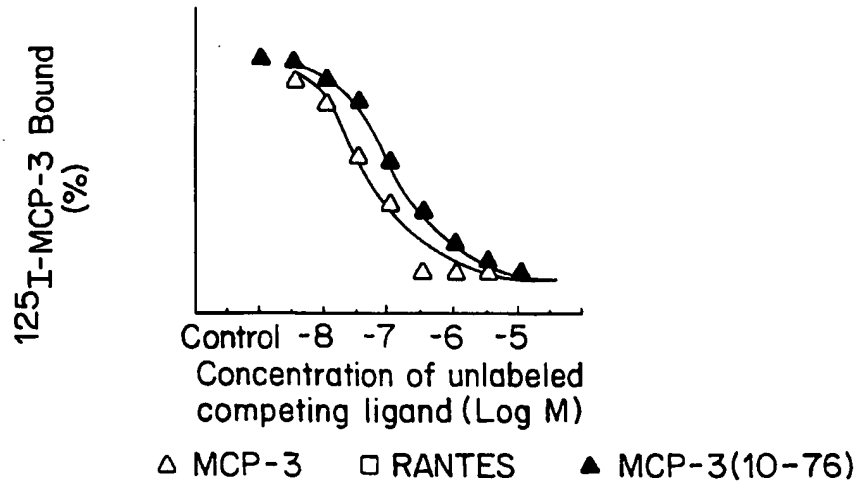

^{125}I-MCP-3 Bound (%)
Control -8 -7 -6 -5
Concentration of unlabeled competing ligand (Log M)
△ MCP-3 □ RANTES ▲ MCP-3(10-76)

FIG. 4E

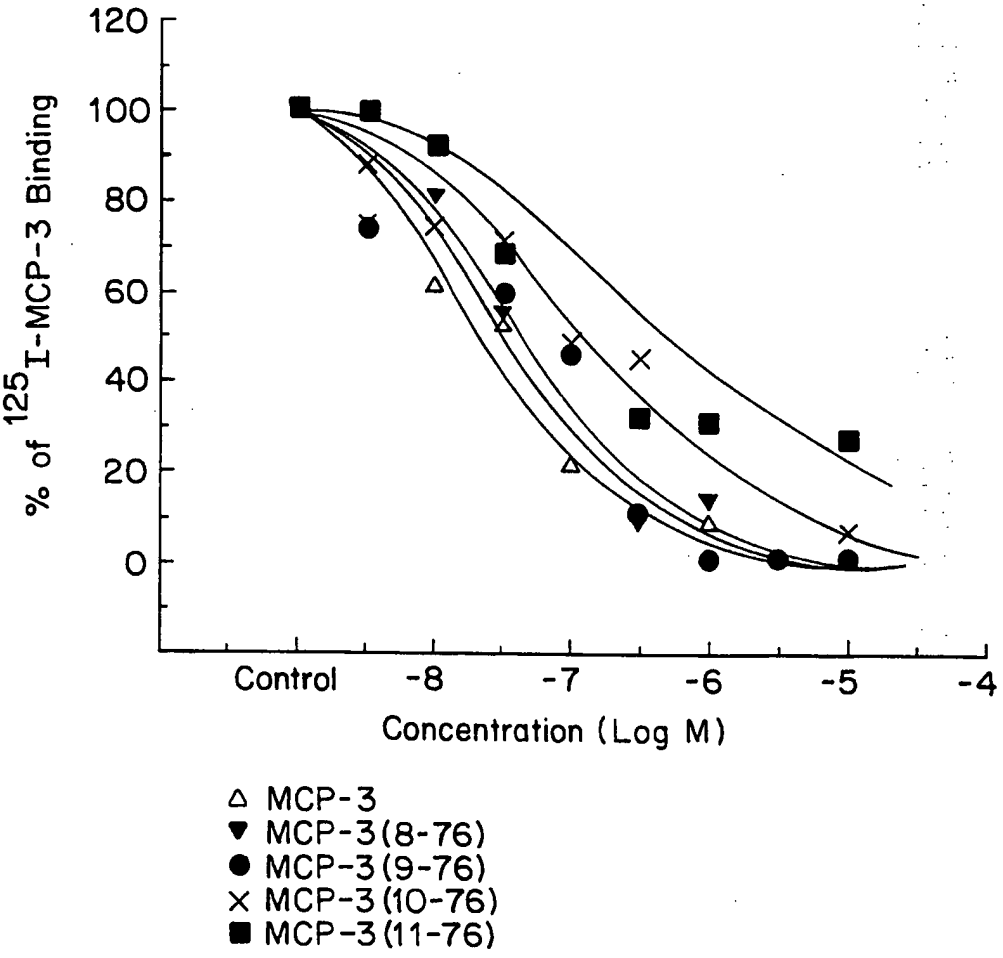

120
100
80
60
40
20
0
% of 125I-MCP-3 Binding
Control
-8
-7
-6
-5
-4
Concentration (Log M)
△ MCP-3
▼ MCP-3(8-76)
● MCP-3(9-76)
× MCP-3(10-76)
■ MCP-3(11-76)

FIG. 4F

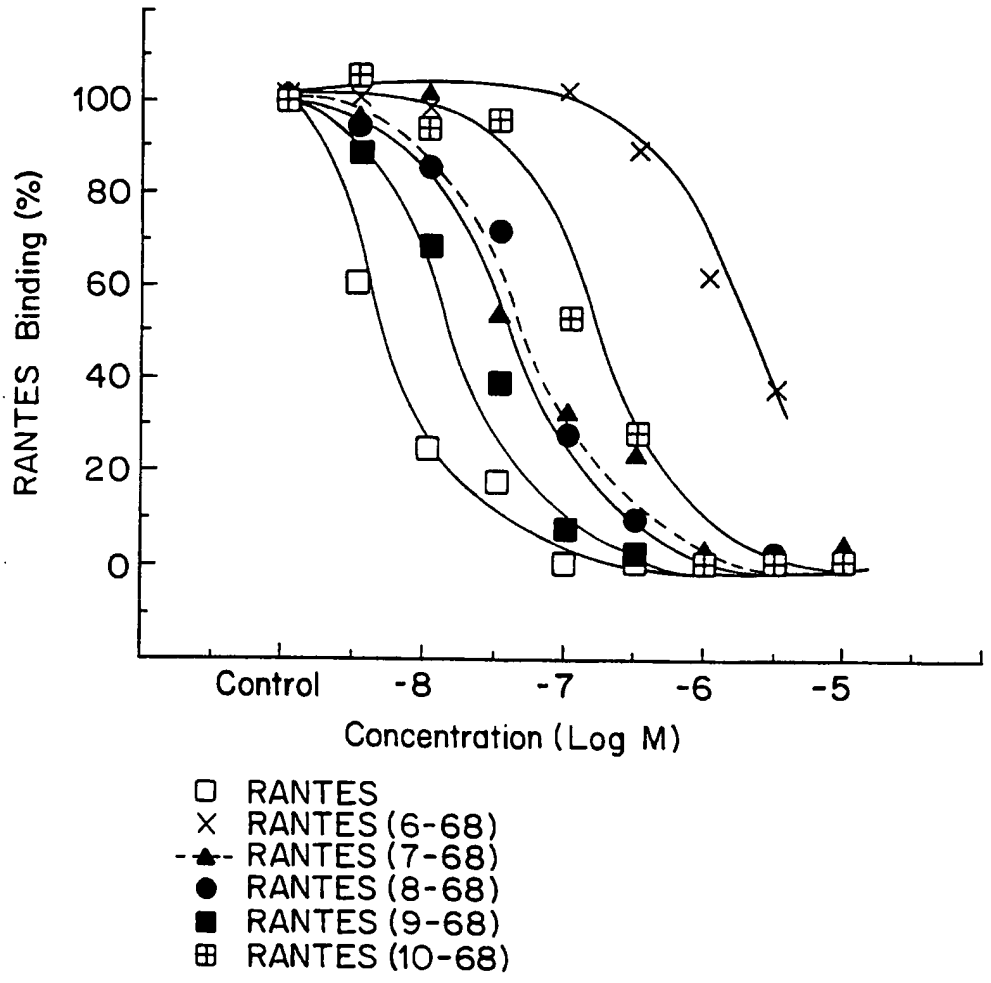

RANTES Binding (%)
100
80
60
40
20
0
Control
-8
-7
-6
-5
Concentration (Log M)
RANTES
RANTES (6-68)
RANTES (7-68)
RANTES (8-68)
RANTES (9-68)
RANTES (10-68)

FIG. 5

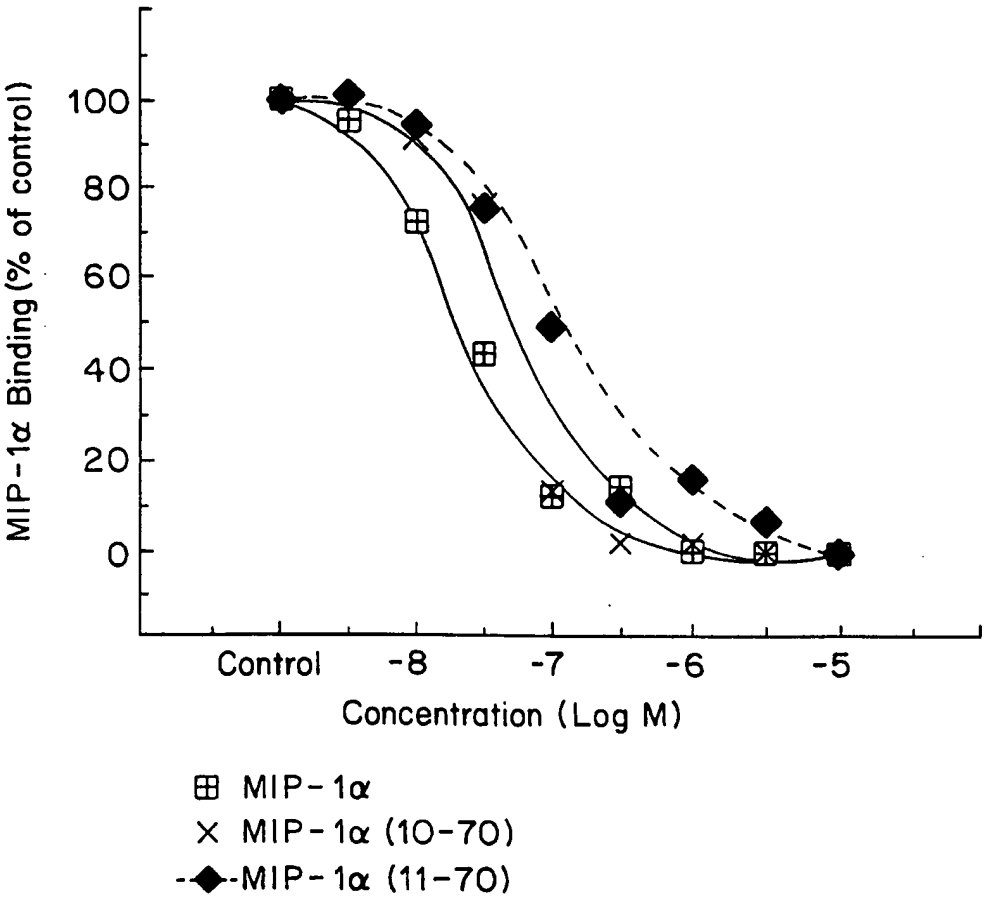

MIP-1α Binding (% of control)
100
80
60
40
20
0
Control
-8
-7
-6
-5
Concentration (Log M)
MIP-1α
MIP-1α (10-70)
MIP-1α (11-70)

FIG.6A

Chemokines or Antagonists	MIP-1α	RANTES	MCP-3
MIP-1α			
Binding (Kd)*	5	10	437
MIP-1α (10-70)			
Binding (Kd)*	25	14	281
Chemotaxis (IC_{50})**	56	100	800
N-Acetyl-β-Glucosaminidase Release (IC_{50})***	1	1	>1,000
MIP-1α (11-70)			
Binding (Kd)*	82	33	No Binding
Chemotaxis (IC_{50})**	560	794	>1,000
N-Acetyl-β-Glucosaminidase Release (IC_{50})***	30	10	>1,000
RANTES (1-68)			
Binding (Kd)*		3 ± 1	No Binding (Kd>500)

FIG.6B

RANTES (9-68)			
Binding (Kd)*	25	19	57
Chemotaxis (IC_{50})**	126	41	200
N-Acetyl-β-Glucosaminidase Release (IC_{50})***	20	4	170
MCP-3 (10-76)			
Chemotaxis (IC_{50})**		620 ± 330	470 ± 170
N-Acetyl-β-D-Glucosaminidase Release (IC_{50})***		48 ± 14	37 ± 5
Binding (Kd)*		50 ± 5	57 ± 5
MCP-3 (1-76)			
Binding (Kd)*		40 ± 9	28 ± 4

The indicated concentrations of chemokines and their antagonists were in nM.

* The receptor binding study has been done on human monocytic cell line THP-1 cells. The concentration of the ^{125}-labeled chemokines (MCP-3, RANTES or MIP-1α) was 4 nM.

** The Inhibition of chemotaxis was done on THP-1 cells. The concentration of all chemokines was 10 nM.

*** The inhibition of enzyme release (exocytosis function of monocytes) has been done on human peripheral monocytes. The concentration of RANTES and MIP-1α used for these experiments was 30 nM. The concentration of MCP-3 used was 10 nM.

FIG. 7

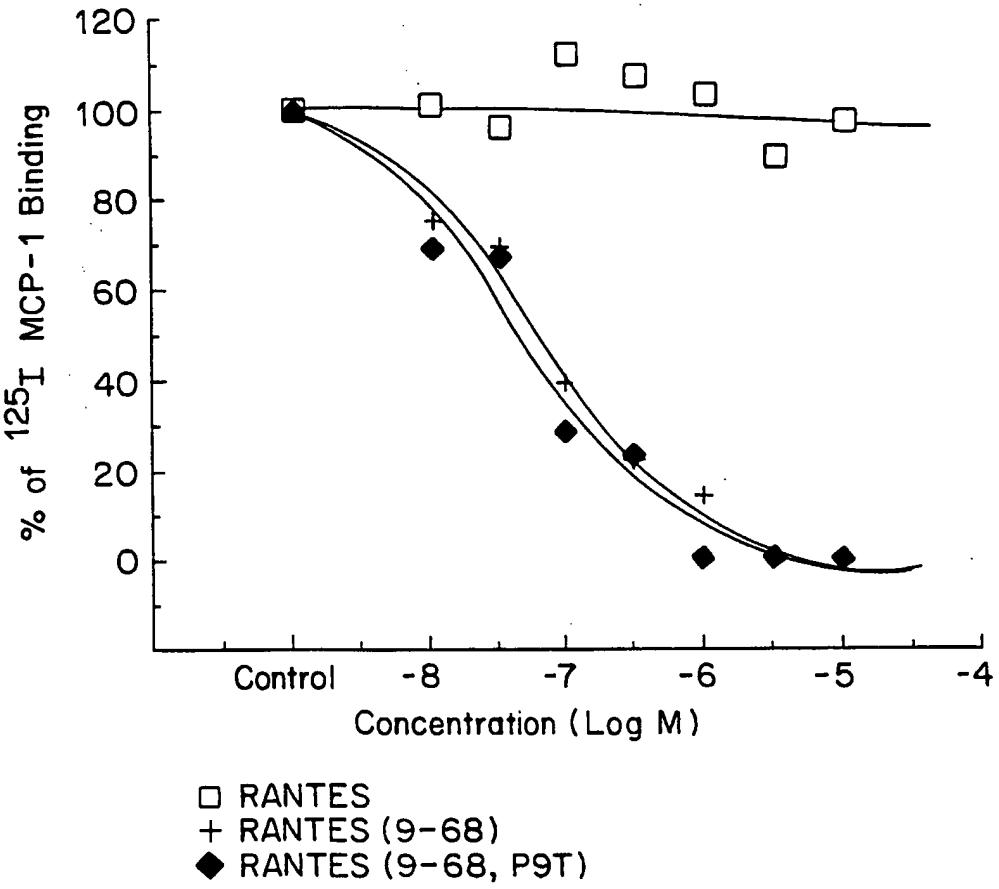

120
100
80
60
40
20
0
% of ^{125}I MCP-1 Binding
Control
-8
-7
-6
-5
-4
Concentration (Log M)
RANTES
RANTES (9-68)
RANTES (9-68, P9T)

FIG. 8

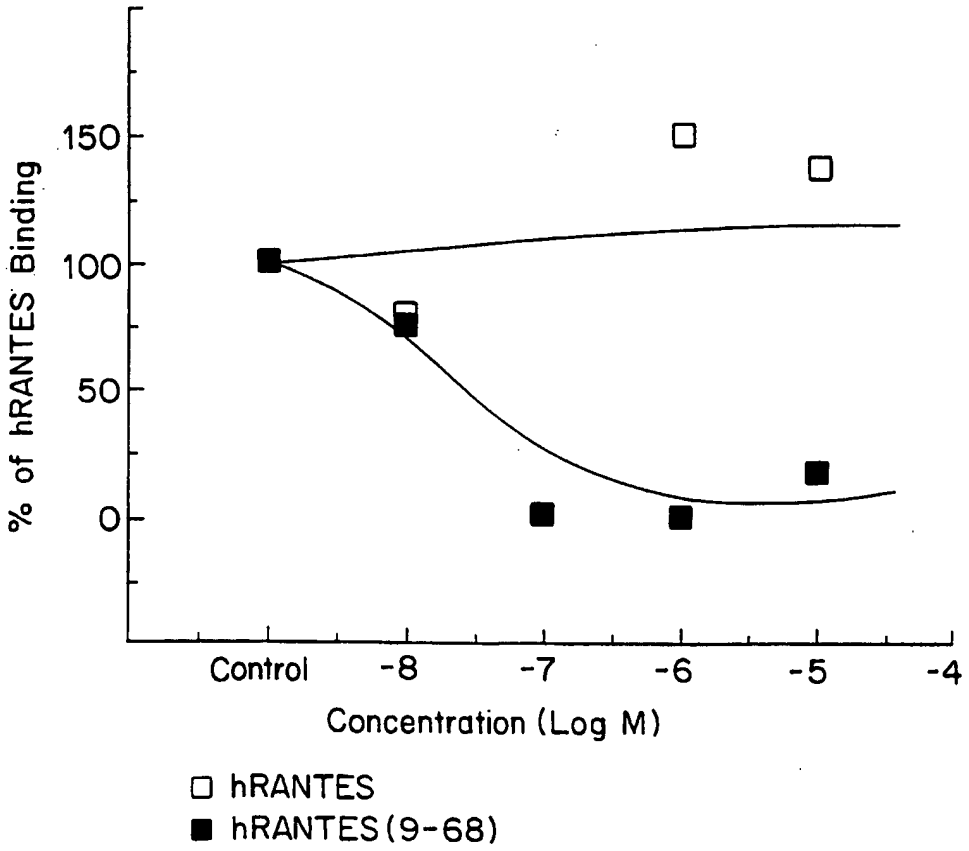

% of hRANTES Binding
150
100
50
0
Control
-8
-7
-6
-5
-4
Concentration (Log M)
hRANTES
hRANTES(9-68)

FIG. 9A

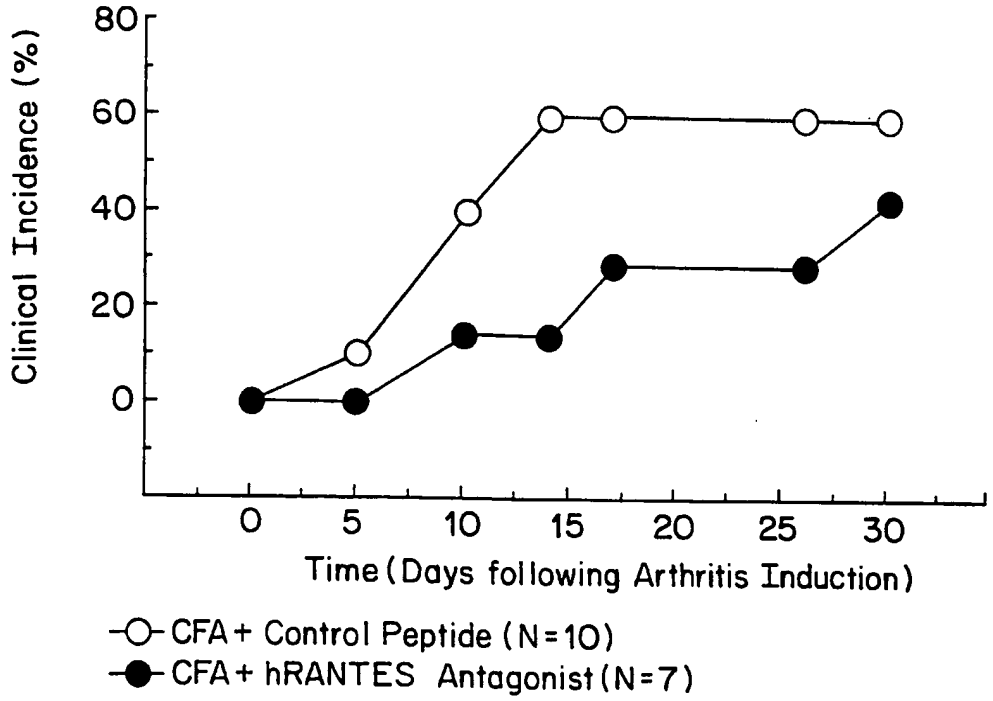

Clinical Incidence (%)
80
60
40
20
0
0
5
10
15
20
25
30
Time (Days following Arthritis Induction)
CFA + Control Peptide (N=10)
CFA + hRANTES Antagonist (N=7)

FIG. 9B

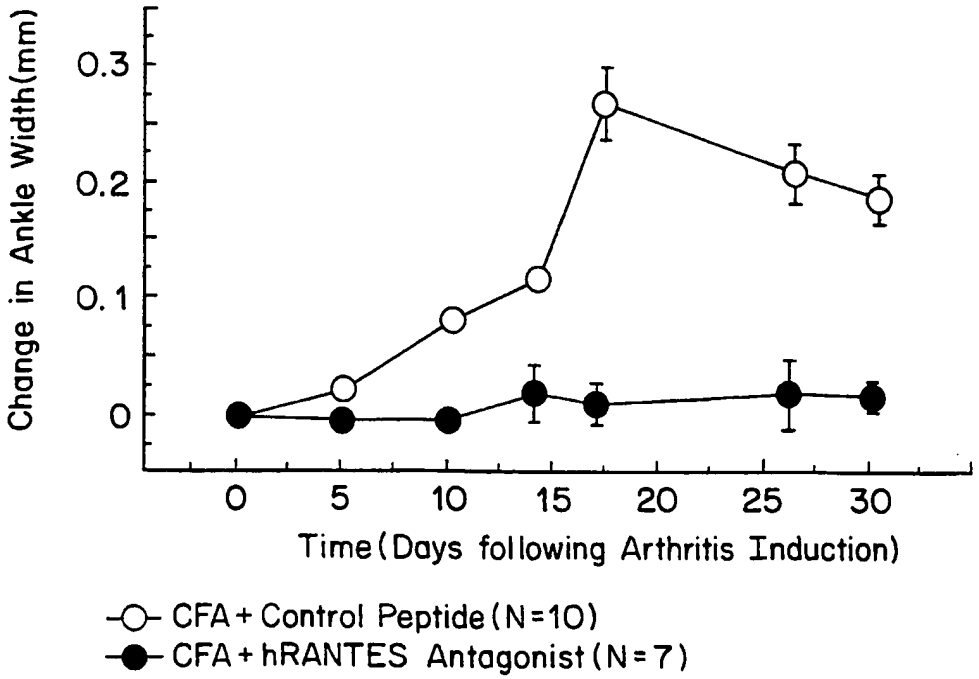

Change in Ankle Width(mm)
0.3
0.2
0.1
0
0
5
10
15
20
25
30
Time (Days following Arthritis Induction)
CFA + Control Peptide (N=10)
CFA + hRANTES Antagonist (N=7)

FIG. 10

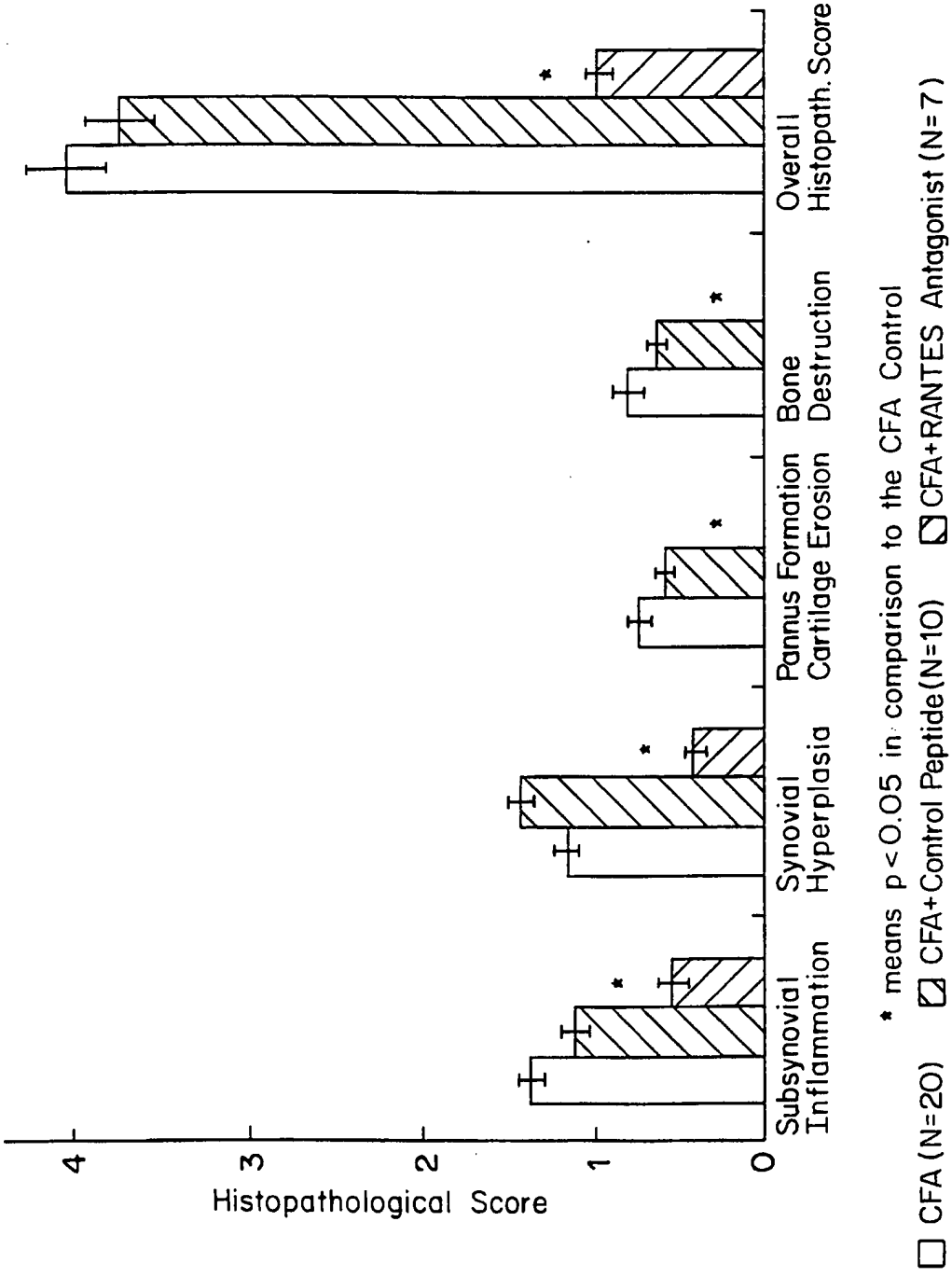

Histopathological Score
0
1
2
3
4
Subsynovial Inflammation
Synovial Hyperplasia
Pannus Formation Cartilage Erosion
Bone Destruction
Overall Histopath. Score
* means p < 0.05 in comparison to the CFA Control
CFA (N=20)
CFA+Control Peptide(N=10)
CFA+RANTES Antagonist (N=7)

FIG. 11

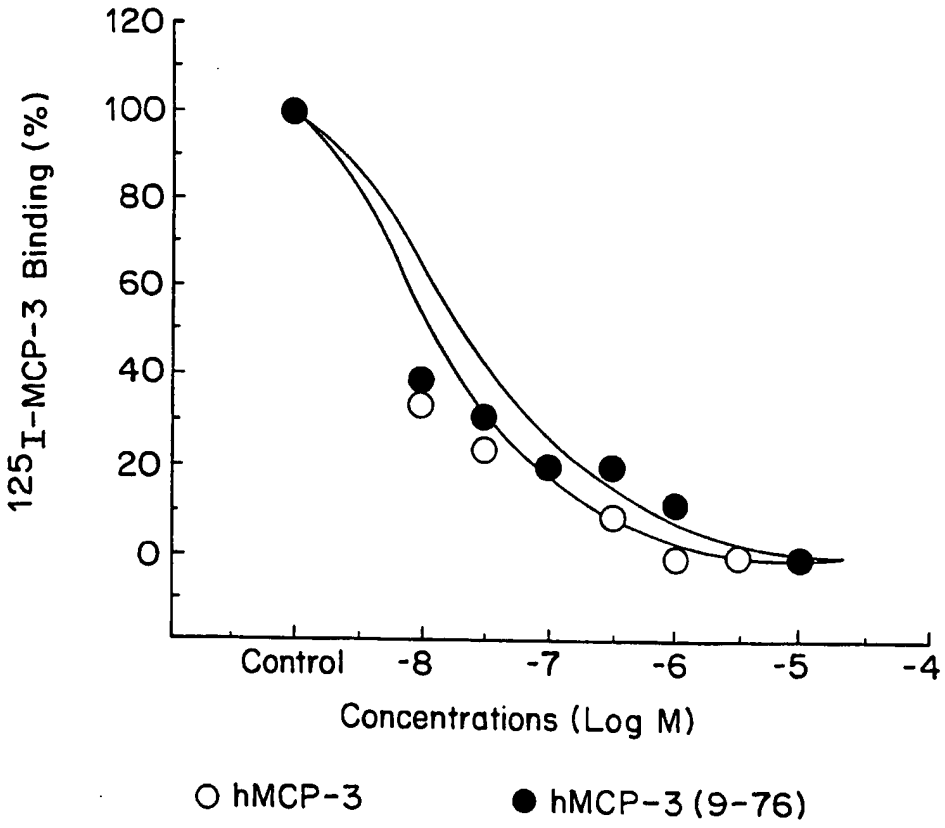
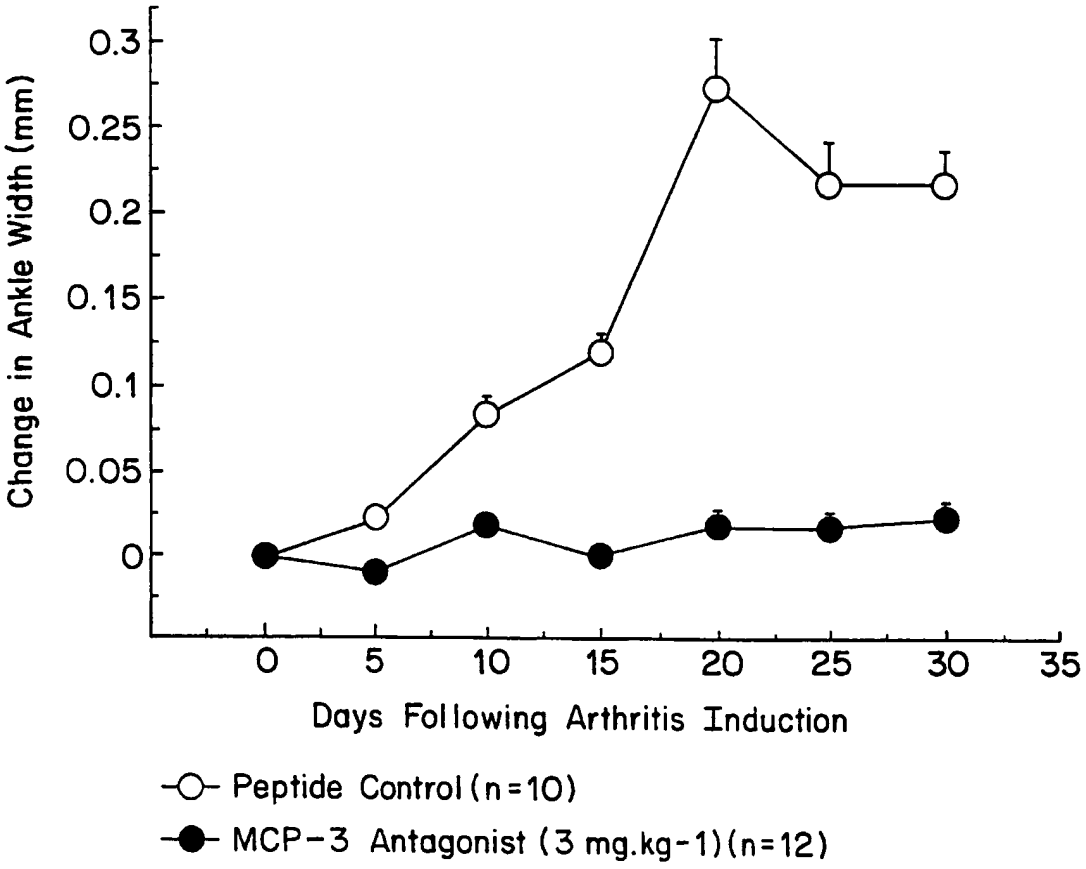

120
100
80
60
40
20
0
125I-MCP-3 Binding (%)
Control
-8
-7
-6
-5
-4
Concentrations (Log M)
O hMCP-3
● hMCP-3 (9-76)

FIG. 12


Change in Ankle Width (mm)
0.3
0.25
0.2
0.15
0.1
0.05
0
0
5
10
15
20
25
30
35
Days Following Arthritis Induction
Peptide Control (n=10)
MCP-3 Antagonist (3 mg.kg-1)(n=12)

INTERNATIONAL SEARCH REPORT

Intern. .ial Application No
PCT/US 97/14485

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K14/52 A61K38/19

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GONG E.A.: "RANTES and MCP-3 antagonists bind multiple chemokine receptors" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 271, no. 18, 3 May 1996, MD US, pages 10521-10527, XP002047804 see the whole document ---	1-22
X	CLARK-LEWIS I ET AL: "STRUCTURE-ACTIVITY RELATIONSHIPS OF CHEMOKINES" JOURNAL OF LEUKOCYTE BIOLOGY, vol. 57, no. 5, May 1995, pages 703-711, XP000605180 See especially pp.709-710 -----	1-22

☐ Further documents are listed in the continuation of box C.

☐ Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
21 November 1997	12.12.97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Groenendijk, M

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 97/14485

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 14-19 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [X] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INVITATION TO PAY ADDITIONAL FEES

International application No.
PCT/US 97/14485

1. Claims: 1-5,14,20(complete), 17-19(partially)

Truncated analogs of MCP-3 as defined in claim 1, their compositions and their use.

2. Claims: 6-11,15,21(complete), 17-19(partially)

Truncated analogs of RANTES as defined in claim 6, their compositions and their use.

3. Claims: 12,13,16,22(complete), 17-19(partially)

Truncated analogs of MIP-1alpha as defined in claim 12, their compositions and their use.